THE
BIOLOGICAL BULLETIN

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CONTENTS

No. 1. FEBRUARY, 1930

PAGE

HÖBER, RUDOLPH
The First Reynold A. Spaeth Memorial Lecture. The Present Conception of the Structure of the Plasma Membrane..... 1

MONTGOMERY, HUGH
The Copper Content and the Minimal Molecular Weight of the Hemocyanins of Busycon canaliculatum and of Loligo pealei 18

FAURÉ-FREMIET, E.
Growth and Differentiation of the Colonies of Zoöthamnium alternans (Clap. and Lachm.) 28

HALL, F. G., and ROOT, R. W.
The Influence of Humidity on the Body Temperature of Certain Poikilotherms 52

MORGAN, T. H., and TYLER, ALBERT
The Point of Entrance of the Spermatozoön in Relation to the Orientation of the Embryo in Eggs with Spiral Cleavage..... 59

LUTZ, BRENTON R.
The Effect of Low Oxygen Tension on the Pulsations of the Isolated Holothurian Cloaca 74

ALPATOV, W. W.
Phenotypical Variation in Body and Cell Size of Drosophila melanogaster 85

JACOBS, M. H.
Osmotic Properties of the Erythrocyte. I. Introduction. A Simple Method for Studying the Rate of Hemolysis 104

No. 2. APRIL, 1930

HOADLEY, LEIGH
Some Effects of $HgCl_2$ on Fertilized and Unfertilized Eggs of Arbacia punctulata 123

WHITAKER, DOUGLAS, and MORGAN, T. H.
The Cleavage of Polar and Antipolar Halves of the Egg of Chaetopterus 145

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8

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PAGE

REDFIELD, ALFRED C.
The Absorption Spectra of Some Bloods and Solutions Containing Hemocyanin 150

CONKLIN, CECILE
Anoplophrya marylandensis n. sp., a Ciliate from the Intestine of Earthworms of the Family Lumbricidae 176

DEMPSTER, W. T.
The Growth of Larvae of Ambystoma maculatum under Natural Conditions 182

SMITH, DIETRICH C.
The Effects of Temperature Changes upon the Chromatophores of Crustaceans 193

No. 3. JUNE, 1930

COE, WESLEY R.
Unusual Types of Nephridia in Nemerteans 203

GRAY, I. E., and HALL, F. G.
Blood Sugar and Activity in Fishes with Notes on the Action of Insulin 217

BLUM, HAROLD F.
Studies of Photodynamic Action. I. Hemolysis by Previously Irradiated Fluorescein Dyes 224

REDFIELD, ALFRED C.
The Equilibrium of Oxygen with the Hemocyanin of Limulus polyphemus determined by a Spectrophotometric Method..... 238

HOADLEY, LEIGH
Polocyte Formation and the Cleavage of the Polar Body in Loligo and Chaetopterus 256

PICKFORD, GRACE EVELYN
The Distribution of Pigment and other Morphological Concomitants of the Metabolic Gradient in Oligochaets 265

SIVICKIS, P. B.
Distribution of Setae in the Earthworm, Pheretima benguetensis Beddard 274

JAHN, THEODORE L.
Studies on the Physiology of the Euglenoid Flagellates. II. The Autocatalytic Equation and the Question of an Autocatalyst in Growth of Euglena 281

PAGE

HARVEY, ETHEL BROWNE
The Effect of Lack of Oxygen on the Sperm and Unfertilized Eggs of Arbacia punctulata, and on Fertilization 288

RAFFEL, DANIEL
The Effect of Conjugation within a Clone of Paramecium aurelia 293

SMITH, GEORGE MILTON
A Mechanism of Intake and Expulsion of Colored Fluids by the Lateral Line Canals as Seen Experimentally in the Goldfish (Carassius auratus) 313

VATNA, SUP.
Rat Vas Deferens Cytology as a Testis Hormone Indicator and the Prevention of Castration Changes by Testis Extract Injections 322

STUNKARD, H. W., and NIGRELLI, R. F.
On Distomum vibex Linton, with Special Reference to its Systematic Position 336

CHAMBERS, ROBERT
The Manner of Sperm Entry in the Starfish Egg 344

INDEX 370

Vol. LVIII, No. 1 FEBRUARY, 1930

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY
Woods Hole, Massachusetts

THE FIRST REYNOLD A. SPAETH MEMORIAL LECTURE[1]

The Present Conception of the Structure of the Plasma Membrane

RUDOLPH HÖBER

Physiologisches Institut, Kiel

Ladies and gentlemen: I feel in this moment that more than ever since the beginning of my scientific life, I have sympathies with this country, where the modern view of general physiology, to which I myself have devoted my life's work, has been developed with perhaps greater success than anywhere else. More than ever here in Woods Hole I feel the genius of Jacques Loeb, who, as no one else since the days of Claude Bernard, taught us so impressively that the most important task of physiology lies in recognizing the general properties of living matter, and who spent here in this place the happiest days of his life doing research work. And sadly here too I remember at this hour my friend, Reynold Spaeth, in whose memory I have the honour to give you this lecture today. Sixteen years ago he came to Kiel with his young wife, an enthusiastic young scientist, eagerly longing to take up physical chemistry as his weapon with which to advance into the undiscovered land of science. And then after his return from Germany, like his great idol Jacques Loeb and many of Loeb's students, he learned to love above all the scientific atmosphere of Woods Hole, —he who was destined to leave us so early, disregarding in his intense eagerness for research the dangers of the tropics.

The *genius loci* of Woods Hole, who apparently holds his protecting hand over general physiology with particular kindness, also moves me to take as the theme of this lecture the present conception of the structure of the plasma membrane. I am sure that with this theme

[1] Delivered at the Marine Biological Laboratory, Woods Hole, on September 9, 1929. The announcement of the foundation of the Spaeth Memorial Lecture will be found in the Report of the Director of the Laboratory for 1928 (*Biol. Bull.*, 1929, **57**: 22).

I shall enter the sphere of interest of many people who have performed and are still performing physiological studies at Woods Hole. At once, with this theme I recall to mind the investigations which Reynold Spaeth put forward with so much skill, perseverance and enthusiasm during his short residence in my laboratory.

Ladies and gentlemen, many of you will agree with me that the problem of cell permeability belongs among the most urgent questions of general physiology, I daresay perhaps of special physiology too. For the working out of a theory of permeability is intimately joined with the understanding of many fundamental phenomena of life, such as nutrition, secretion, absorption, excretion, growth and irritability. Hence let us begin to follow a little the development of the doctrines of the permeability of cells.

In 1855 Naegeli described the phenomenon of plasmolysis of the plant cell, consisting in a persisting retraction of the protoplast from the cell wall, if the cell is bathed in what we call today a hypertonic solution. Pfeffer in 1877 gave an explanation of the permanent plasmolysis, comparing the plant cell with the "Traubesche Zelle" made up by a precipitation membrane, for instance, by a copper ferrocyanide membrane. He suggested that the protoplast is surrounded by some limiting layer on the outer surface, the plasma membrane having, like the inorganic precipitation membrane, the property of semipermeability, that is, permeability to water but impermeability to such dissolved substances as produce the plasmolysis. Some time later, Overton gave an unquestionable proof that Pfeffer's assumption was correct; making use of a series of organic compounds, he showed that, in full harmony with the theory of solutions based by van't Hoff upon the experiments of Pfeffer, all solutions which produce the beginning of plasmolysis have the same molecular concentration. Thus at first one was compelled to assume that the interior of the living cell was shut off from dissolved substances and that only water was able to enter.

The next important step in recognizing the nature of the limiting membrane of the cell was the discovery of Klebs and de Vries that besides the dissolved substances which cause permanent plasmolysis, there exist some others,—for instance glycerol and urea, which given in hypertonic solution, instead of bringing about the permanent plasmolysis, only produce the initiation of shrinking, which is followed sooner or later by deplasmolysis. Furthermore, Overton and others have discovered a great many substances that do not plasmolyse at all. It was only a logical outcome of the theory of the plasma membrane to explain all this by the assumption that for such substances the mem-

brane is not impermeable, but allows them to pass faster or slower. These conclusions could be often established beyond doubt by chemical, optical and other forms of analysis of the contents of the cells.

But the question now arises as to whether the inorganic precipitation membranes behave in perhaps the same manner, that is, whether or not they are permeable to the same substances to which the plant cells, as indicated by the plasmolysis experiments, are permeable. Curiously enough, this question, which is derived so easily from the experiments, has been answered only recently by the systematic experiments of Collander.[2] This author showed that the copper ferrocyanide membrane behaves also in a very different manner in relation to a great number of organic non-electrolytes, allowing some to pass not at all, others to pass slowly, and still others quickly. But the laws governing the speed of permeation through the precipitation membrane differ widely from those which hold good in the case of the plasma membrane, as is illustrated by Table I.

TABLE I

Substance	Relative permeability of *Rhœo discolor*	Permeability of Copper Ferrocyanide	Molecular Volume	Relative Solubility in Ether
Methyl alcohol	125	+++++	8.2	0.273
Ethyl alcohol	71	++++	12.8	1.86
Valeramide	69	++	28.7	0.170
Ethyl urethane	59			0.637
Ethylene glycol	4.4	++++	14.4	0.0068
Diethylurea	2.0			0.0185
Glycerol	1.3	+++	20.6	0.0012
Methylurea	1.2			0.0012
Urea	1.1	+++++	13.7	0.0005
Glucose	1.02	+	37.5	<0.0001
Glycocoll	1.0	++++	17.1	<0.0001
Saccharose	1.0	+	70.4	<0.0001

The table contains data with respect to the behavior of twelve organic non-electrolytes. The first series of numbers shows the various speeds of permeation as related to the epidermis cells of *Rhœo discolor*, the second the speeds of permeation in relation to the copper ferrocyanide membrane. It can be easily seen that between both there exists no parallelism at all. The third series indicates the molecular volumes calculated by Collander from the values of molecular refraction; the fourth series gives their relative solubilities in ether. Now comparing the second and third series, we recognize clearly that the velocity of permeation of the precipitation membrane is a function

[2] Collander, *Kolloidchem. Beihefte*, **19**, 72, 1924 and **20**, 273, 1925.

of molecular volume. This governing rule being established, the character of the membrane is immediately revealed. It behaves as a sieve for molecules so that the size of its pores determines whether or not the dissolved substance can permeate. Such a membrane is semipermeable as soon as the diameter of the molecules of the solution surpasses a certain size. The passage is then allowed only to water, for its molecules are characterized by an especially small volume. And since it is highly probable that the pores of the membrane are not all of the same size, the molecules with a diameter below the limiting value have a greater chance to slip through, as they are smaller. Furthermore, the fourth series of numbers shows that the permeability of the plasma membrane might depend upon quite another principle, that is, the principle of solubility in the substance of which the membrane is composed or, briefly, the principle of selective solubility. Thus we come to speak of the first comprehensive theory of cell permeation, the lipoid theory of Overton.

It is a well-known fact that the lipoid theory has been supported by a large amount of powerful arguments, but it is also well-known that one has struggled sharply against it, very often, I believe, with insufficient arguments. But even today the theory cannot conclusively be judged for the simple reason that the physico-chemical foundation is partly too narrow and partly too uncertain. Professor Jacobs was indeed completely right when he wrote a short time ago: "It may be emphasized that what is most needed in the field of cell permeability at the present day is facts." As everybody knows, Overton based his theory in the first place on the comparison between the speed of penetration of substances and their relative solubility in oil. Collander, recently reviewing most carefully the experiments of Overton on plant cells, advocated especially the correspondence between permeability and solubility in ether.[3] Both these authors are quite clear about the limited value of their comparison, and the table also shows that the parallelism is fairly incomplete. Therefore the lipoid theory is still nowadays a *petitio principii*. However, the thesis that the permeability to the organic non-electrolytes is to be compared to the solubility in organic solvents agrees so often with the experimental data, that I myself have practically no doubt that it is only necessary to discover such solvents as might be still better suited to comparison with the material of the plasma membrane than oil or ether. It is really astonishing that since the lipoid theory was set up more than thirty years ago, so little systematic research work

[3] Collander and Bärlund, *Soc. scient. fenn.*, **2**, 9, 1926; Bärlund, *Acta botan. fenn.*, **5**, 1929.

has been done on the relative solubility of organic compounds in different organic solvents comparable to the lipoids of Overton, in order to get a firm basis for the theory. It is well known that an interesting attempt to find a better model was made by Nirenstein some years ago.[4] He showed that several exceptions to the rule previously given by Paul Ehrlich, that the vital colors which enter easily into the living cell dissolve in oil, could be removed by trying to imitate the plasma membrane with a mixture of an oil with a fatty acid and an organic fat-soluble base. Table II shows experiments by which I was able to compare the relative solubility of acid dyes in the above-mentioned oil mixture with the relative absorption of colors by red blood corpuscles.[5]

TABLE II

Sulfonic Acid Dyes	Relative Solubility in Oil Mixture	Relative Absorption by Blood Corpuscles	Sulfonic Acid Dyes	Relative Solubility in Oil Mixture	Relative Absorption by Blood Corpuscles
Wollgrun, Lichtgrun, Cyanol, Eriocyanin	0	0	Tropæolin 1	30	1–3
			Tropæolin 2	30	4–8
			Orange R	85	7–16
Azofuchsin I	< 1	0.3–1	Brilliant orange R	71	7–16
Azofuchsin G	< 1	<1	Metanil yellow	94	10–16
Bromophenol blue	27	1–3			

It can be seen that there exists a parallelism between solubility and absorption, and it is especially noteworthy that this similarity means not only intensity of staining, but means permeability; for it follows from the table that dyes which are not dissolved in the oil mixture do not enter the blood corpuscles at all. However, such experiments with dyestuffs do not come out quite satisfactorily, as I can easily show. Therefore it is necessary to collect further experimental data to get a clear understanding, inasmuch as the cell permeability is a solution permeability.

But there can be no doubt that the cell permeability is not only a solution permeability with regard to an oil-like solvent within the plasma membrane. In the first place in this connection it is a very striking fact that water enters the cell usually with remarkable speed, because it is impossible to reconcile this entrance with the supposition that the membrane consists entirely of an oily phase. Secondly there exists an apparent permeability of certain kinds of cells to inorganic anions, though the inorganic salts are generally not

[4] Nirenstein, *Pflüger's Arch.*, **179**, 233, 1920.

[5] Unpublished experiments.

in the least soluble in organic solvents. Thirdly, there are important nutritive materials which cannot get into the cell in any way, but belong also to those substances that are nearly or entirely insoluble in the organic solvents.

Now we are able to interpret the first and second points by returning to the already-mentioned sieve theory of permeability of the precipitation membranes, and we will see that on that account the comprehension of the structure of the plasma membrane receives a very important supplement. More than thirty years ago Koeppe, Gürber and Hamburger made the discovery,—which has often been verified since,—that the red blood corpuscles have a selective permeability for anions. It is well known that this property has the greatest importance for the buffer capacity of the blood; but it seemed for a long time to be a strange *unicum*, for which there existed hardly any physical parallel. Otherwise it might have been possible to construct a model to imitate the peculiarities of the membrane of the blood corpuscles. Today the matter is practically clear; the well-known experiments of Michaelis and Collander with artificial membranes, especially with dried collodion membranes, enabled us to understand the singular phenomenon. Michaelis proved that these membranes are, under certain circumstances, the seat of a great potential difference, whose direction and amount is an obvious sign that the membranes are exclusively cation-permeable.[6] Therefore there is an analogy between the cation-permeable collodion membrane and the anion-permeable blood corpuscle membrane. At the collodion membrane the anion plays no rôle, whereas a cation gives rise to an electromotive force which increases as its migration velocity increases or as its diameter decreases. On account of these facts Michaelis has proposed the following hypothesis: the membrane allows only the cation to pass through it as through a sieve; the ions with the smallest diameter pass with the greatest speed, and the entrance of ions into the pores of the membrane is prevented if their diameter exceeds a certain value. That is apparently the reason why, for instance, the earth-alkali ions are unable to pass some collodion membranes characterized by rather narrow pores. In this way we may understand that a quantity of an ion sufficient to be detected by chemical methods can penetrate only if there is present another cation on the other side of the membrane, so that an exchange can take place. That is exactly the same as with the red blood corpuscles, where from the beginning the demonstration of the selective anion-permeability depended upon the fact that as long as there exist differences of concentration in the proper

[6] Michaelis, *Naturwissenschaften* 1926, **14**: 33.

direction, the anions of the surrounding solution can be exchanged against the anions of the interior of the cell.

And now the question arises, how it is to be understood, that in the case of the collodion membrane the pore permeability is limited to the cations and, in the case of the red blood corpuscles, to the anions. Michaelis had already turned his mind to the fact that the substance of the cation-permeable membrane itself is negatively charged, and he connected this idea with the well-known membrane studies of Bethe and Toropoff [7] and the experiments on the reversal of membrane potentials in gelatine discs, which have been established by Matsuo in my own laboratory.[8] As a matter of fact it can be proved that this idea is right. There exists a relation between the electric charge of the membrane material and the faculty of the ions with opposite electric charge to pass. In my laboratory Mond succeeded in demonstrating that if the negative charge of the collodion is changed to a positive charge by addition of a basic dye, for instance by rhodamin, the membrane thus formed, instead of being exclusively cation-permeable is changed into a membrane of selective anion-permeability.[9] Table III illustrates the resulting conditions.

TABLE III

Membrane Potentials in Rhodamin-collodion Membranes

	———→ Cl			
0.1M NaCl	———→	0.1M NaSCN	+ 60	millivolts
0.1M NaCl	———→	0.1M NaI	+ 33	"
0.1M NaCl	———→	0.1M NaBr	+ 20	"
0.1M NaCl		0.1M NaCl	0	"
0.1M NaCl	←——	0.1M Na_2SO_4	− 3.8	"
0.1M NaCl	———→	0.1M KCl	+ 2	"

$SCN > I > Br > Cl > SO_4$

Cations without effect

The dotted arrows show the direction of the movement of the chlorine ions; their length is a measure of the potential dependent on the velocity of the ions. The arrows drawn refer in a corresponding manner to the anions of the opposite side of the membrane. The electromotive forces decrease from + 60 millivolts to − 3.8 millivolts along the series of anions: thiocyanate, iodine, bromine, chlorine, sulfate. The cations are without any effect. The membrane potential is therefore approximately zero if there is sodium chloride and

[7] Bethe and Toropoff, *Zeitschr. f. physik. Chemie*, **88,** 686, 1914 and **89,** 597, 1915.

[8] Matsuo, *Pflüger's Arch.*, **200,** 232, 1923.

[9] Mond and Hoffmann, *Pflüger's Arch.*, **220,** 194, 1928.

potassium chloride in the same concentration on each side of the membrane.

There can be no doubt that these experiments demonstrate on the one hand in a very conclusive manner the existence of anion-permeability on a membrane originally cation-permeable, but they reveal on the other hand some difficulties in our understanding of these and, as we shall see, of other alterations of the ion-permeable membranes. The membrane potential of the rhodamin collodion membrane does not increase with increasing migration velocity of the effective ion, as has been found by Michaelis with the cation-permeable collodion membrane, but the potential changes according to the lyotropic series. This seems to point to some kind of relation of ion-permeability to the colloidal state of the membrane, which is known to depend in an especially characteristic manner on the lyotropic properties of the ions.

However, before discussing this question more amply, we will look at a remarkable consequence of the membrane experiments just described. Mond, supposing that the membrane material of the red blood corpuscles is electropositive, suggested that their natural anion-permeability might be turned into cation-permeability, if one succeeds in giving the membrane substance a negative charge.[10] This actually happens by the addition of a suitable amount of hydroxyl ions. As soon as the reaction in the surrounding medium of the blood corpuscles is made more alkaline than pH 8, the usual selective anion-permeability is displaced by selective cation-permeability, so that now an exchange between the potassium ions of the interior with the sodium ions of the environmental solution begins, while the chlorine and bicarbonate ions present in both serum and corpuscles, which were up to this point able to pass through, are now fixed. Mond has advocated the view that the decisive constituent of the plasma membrane, to which the opposite charge is to be attributed, has ampholyte character and might be globine, that is, a protein body, because the reaction by which this reversal of anion-permeability into cation-permeability takes place conforms with the isoelectric point of the globine, which is pH 8.1.

In this way we come to a conception, similar to the well-known hypothesis of Nathansohn, that the cell surface is comparable to a mosaic of both lipoids and proteins. Apparently the plasma membrane of the red blood corpuscles consists of at least two constituents, a lipoid phase, whose existence enables the lipoid-soluble substances to enter, and a protein phase, which is pore-permeable, so that water

[10] Mond, *Pflüger's Arch.*, **217**, 618, 1927.

as well as dissolved substances, whose molecular size is small enough, can pass through. As to the character of the structure of the cation-permeable membranes, which we will now discuss, the opinion is not yet substantiated enough.

TABLE IV

Resting Potentials of the Sciatic Nerve

Time	Potential	Solution	Time	Potential	Solution
	millivolts			*millivolts*	
3:21	20.6	Ringer	3:20	27.4	Ringer
3:53	20.5	Ringer	3:52	27.5	Ringer
3:55		Ringer with 0.08% KCl	3:54		Ringer with 0.08% KCl + 0.1% $CaCl_2$
4:03	19.0		4:02	27.7	"
4:28	17.2		4:27	28.5	"
5:18	16.5		5:17	28.6	"

It seems that the cation-permeable membranes exist more frequently than anion-permeable membranes. As Bernstein and I have pointed out twenty-five years ago, the hypothesis of selective cation-permeability gives a good explanation of the electro-negativity that results from injury and of negativity resulting from activity. But if we produce a difference of potential in uninjured tissues such as muscles, liver and apple by joining their surfaces at two different points with two different salt solutions, then we see that, although the cations are better enabled to produce an electric current (apparently in connection with the negative character of the membrane colloids), the anions have an action too, the strength of which is correlated with their position in the lyotropic series: sulfate, chlorine, bromine, nitrate, iodine, thiocyanate. But the cations also do not act exactly according to the series of the size of the ions: cæsium, rubidium, potassium, sodium, lithium, but succeed each other as potassium, rubidium, cæsium, sodium, lithium,—a series met with rather often, as I have found in relation to changing the hydrophilic colloidal state, and characterized by the peculiar dislocation of cæsium. And finally, regarding the membrane potentials of muscle and nerve, we encounter a cation antagonism, for example that between potassium and calcium, which might be explained by assuming an influence on the state of membrane colloids, whereas it is difficult to explain it by supposing the existence of a sieve-like membrane, the size of whose pores remains unchangeable. Table IV gives as an example the behavior of the sciatic nerve of a frog.[11]

[11] Höber and Strohe, *Pflüger's Arch.*, **222**, 71, 1929.

As the experiment on the left side demonstrates, the resting potential falls if the uninjured surface of the nerve is brought into contact with a Ringer's solution in which the percentage of potassium chloride is raised to more than 0.08. If we increase not only the concentration of potassium chloride but also of calcium chloride to 0.01, the alteration of the initial potential, as is to be seen in the experiment on the right side of the figure, does not take place. So we notice again in regard to the membrane potentials the well-known antagonism between potassium and calcium, and since there is hardly any doubt that the permeability of the plasma membrane due to its porous structure plays a significant rôle, we concluded that this permeability is, according to the nature of the composing material, much more variable than the pore-permeability of the artificial ion-permeable membranes, especially of the collodion membranes.

Before leaving the interesting question of ion-permeability, I wish to direct your attention to a membrane with very curious qualities. Last year I set up and examined a membrane which was a patchwork of cation-permeable pieces of collodion and anion-permeable pieces of rhodamin collodion.[12] Figure 1 gives the scheme. This membrane must have the following qualities, and in fact it does have them. If we place a salt solution on one side of it, for instance, a solution of potassium chloride, and on the other side water, the salt cannot diffuse into the water, although the membrane is as permeable for the potassium ions as for the chlorine ions, because a passage in chemically detectable quantity would be possible only if it could happen at just the same place in equivalent amounts of cation and anion, or in other words, because one ion can move only at an infinitesimal distance from the opposite. However, the passage of the potassium chloride is rendered possible as soon as a salt, whose ions can interchange through the membrane with the potassium and the chlorine ions, is placed on the other side of the membrane. It seems to me that membranes of this kind, which, in spite of their permeability for anion and cation, are able to entirely prevent the escape of salts, have been realized by nature and play an important rôle.

Now the question arises as to whether, in addition to the water, only inorganic ions take the way through the pores of the plasma membrane. Logically the answer is no. For if there are molecules whose volume is of the same order as that of the permeating ions, then they naturally must take the way through the pores, regardless of the possibility of their passing equally well through the membrane by

[12] Höber and Hoffmann, *Pflüger's Arch.*, **220**, 558, 1928.

selective solution. Of course it has been pointed out by Michaelis that the collodion membrane, if it is dried enough to establish selective ion-permeability and therefore to give the maximum electromotive effect, allows those molecules to pass whose diameter is about the same as that

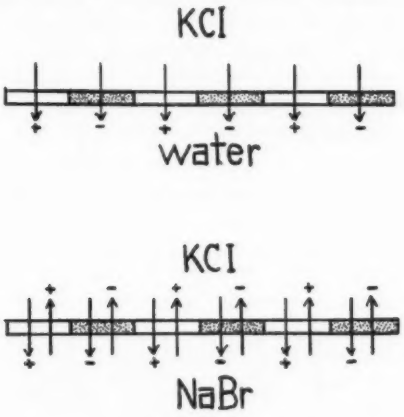

FIG. 1.

of glucose.[13] A similar behavior is met with in the plasma membranes. It will be noted that among the organic non-electrolytes entering into the cell, there are some which permeate more quickly than might be expected in relation to their relative lipoid-solubility, or, more correctly, in respect to their relative solubility in ether, supposing that the relative solubility in ether is to be acknowledged as a likely measure of the physiological phenomenon. Some of these substances are characterized by a relatively small molecular volume, for instance, ethylene glycol and glycerol. Therefore Collander may be quite right in considering their comparatively rapid permeation into plant cells as due to the porosity of the plasma membrane.[14] In other cases where a disagreement occurs between velocity of penetration and solubility in ether, for example with urea and its derivatives, even the view of a sieve-like property fails to overcome the difficulties. But here we can see, as I have found with Watzadse, that the difficulties will be removed if, instead of the solubility in ether, the solubility in the previously mentioned oil mixture of Nirenstein will be correlated with the physiological phenomenon.[15]

[13] *Loc. cit.*

[14] *Loc. cit.*

[15] Watzadse, *Pflüger's Arch.*, **222**, 640, 1929.

The assumption of the porosity of the plasma membrane in this manner being justified in several ways, it will be necessary to study as intimately as possible the properties of the artificial porous membranes and especially, because of their great stability, those of dried collodion membranes. Therefore perhaps it is not too audacious to consider the possibility, in relation to physiological conditions, that certain molecules with a diameter not too great and not too small might be stopped in the pores and obstruct them in the same manner that ultramicroscopic particles are not only kept back by an ultrafilter, but finally also obstruct its pores.

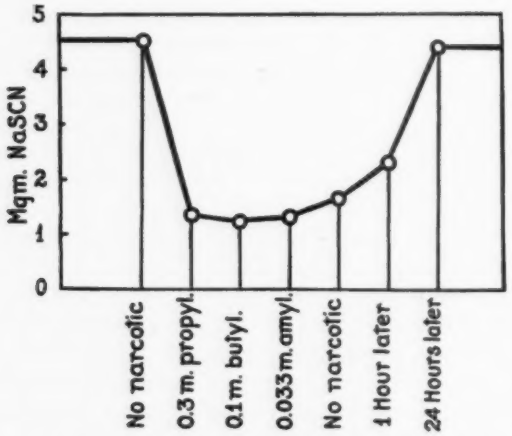

FIG. 2. Diffusion of thiocyanate in 15′, retarded by urethanes.

From this point of view Anselmino has made experiments in my laboratory. He favored the obstruction of the pores by using narcotics, because they can be adsorbed by the collodion.[16] The result was that the collodion membrane was obstructed to such a degree that the osmotic movement of water as well as the diffusion of molecules of small size was strongly retarded. Figure 2 reproduces a striking experiment. You see that the diffusion of thiocyanate is reversibly slowed by several urethanes, and that the homologous urethanes exert their influence characteristically so that the longer their carbon chains are, the smaller their limiting concentration will be, in the same way that we usually observe in narcosis.

[16] Anselmino, *Pflüger's Arch.*, **220**, 524, 1928.

It will be necessary to find out still more exactly which substances are suitable for the obstruction of the pores and which are not. Michaelis has recently found that the speed of diffusion of glucose through a collodion membrane of suitable pore size decreases with time more and more, and he regards this too as effectuated by an obstruction of the pores.[17] But in this case we do not have to deal with an adsorbable substance. It still remains an open question for the future, how far the decrease of cell-permeability during narcosis, so often already observed, is to be attributed to the porosity of the plasma membrane. If this really happens, our ideas as to the nature of narcosis would be greatly supplemented.

Now we will consider an especially difficult matter. As we have seen, the entrance of numerous organic non-electrolytes into the living cell may be considered as a matter of lipoid-solubility; the entrance of other organic non-electrolytes, of some ions and of water may be considered as a matter of diffusion through the pores of the plasma membrane. But there is a group of substances of a very remarkable physiological significance, which can neither directly enter by dissolving in the oily phase nor by migrating through the porous phase, but which, nevertheless, do obviously enter. To this group belong substances which constitute a considerable proportion of the nutritive material, such as many sugars and amino-acids. There can be no doubt that this passage is not merely a simple form of permeation, in the sense that it depends on a certain permanent and invariable physicochemical behavior of a membrane. Either the plasma membrane must change under definite conditions in such a way that a temporary removal of the barrier to diffusion is brought about, or,—as has often been supposed,—reversible chemical reactions of the foodstuffs occur even in the surface of the cell, so that either the products of reaction are enabled to pass through or a more or less complicated series of single reactions is terminated by the appearance of the foodstuffs inside the cell wall.

Adhering to the physicochemical character of this lecture, we will discuss, basing our remarks on experimental data, only one of these forms of the ingestion of the nutritive substances, namely the alteration of the plasma membrane in such a way that for a short time it becomes permeable to substances for which it is otherwise not permeable.

As a matter of fact, there is a well-known form of intake of nutritive material which can be considered as an opening of the plasma membrane, that is, phagocytosis. For as the protoplasm is flowing around the particles of food in order to incorporate them, the superficial layer

[17] Michaelis and Weech, *Jour. of Gen. Physiol.*, **12**, 55, 1928.

necessarily must be partly destroyed. On the other side there exist further conditions for naturally opening the plasma membrane in a reversible manner, particularly as a so-called functional increase of permeability, that is, as an increase of permeability accomplished by function, or better, by excitation as preparation for function. I am not able to give an extended review of our knowledge of functional increase of permeability within the limits of this lecture, but I shall relate one striking demonstration of the bringing about of a reversible increase of permeability. If one brings *Spirogyra* cells into a solution of cyanol, a well-diffusing blue sulfonic acid dye, the protoplasts will remain unstained for several weeks. Some years ago in my laboratory Banus observed that while sending an alternating current of appropriate strength through the threads of algæ, the blue dye would pass out of the solution into the interior of the cell, namely into the sap of the vacuoles.[18] After this, the current being stopped, the algæ were left for some time in the blue solution; then they were taken out and washed with pure water. It resulted that the vacuole retained the blue dye in spite of its diffusibility, the dye which entered being imprisoned as long as the cell was alive. Apparently the electric current had opened the plasma membrane, a substance to which the interior of the cell is closed under natural conditions had penetrated, and behind it the plasma membrane had shut up. In this way an event was produced, owing to experimental conditions, that is never realized in nature; but a natural phenomenon, the reversible increase of permeability, had been reproduced, possibly in a somewhat crude manner. Perhaps there occurred only a regeneration after an injury generated by the current. But, examining the conditions more closely, we may recognize that nature may sometimes duplicate them. For, in regard to the well-known studies of Bethe and Toropoff on gelatine diaphragms, it is highly probable that the flow of an electric current is accompanied by changes of hydrogen and hydroxyl ion concentration on the cell boundary so that these active ions, either by hydration and liquefaction or by aggregation of the surface colloids, can amplify or narrow the paths to be taken by diffusing substances and can in this way produce reversible changes in permeability.

Thus we learn more and more to regard the plasma membrane as a formation with varying properties so that its permeability exhibits different degrees succeeding one another in time. But the plasma membrane does not only vary in one and the same object temporarily, but,—and this shall be the last point to be discussed in this lecture,—it varies also in one and the same kind of cell from species to species.

[18] Banus, *Pflüger's Arch.*, **202**, 184, 1924.

I shall only demonstrate this with one especially simple object, namely, the red blood corpuscles again, and with this object I wish to demonstrate further in what direction the research into the nature of cell permeability is to be extended. Finally I return in this way once more to the phenomena of porous permeability and of solution permeability of the cells.

As we have seen before, the limiting membrane of the blood corpuscles, according to the electropositive charge in the wall of its pores, allows only the anions to exchange by diffusion from one side of the membrane to the other. Further, it has been pointed out by different authors that each anion passes through the corpuscle membrane with a specific velocity. Now Mond in my laboratory has raised the question of the existence of differences in the relative velocities from species to species as evolving from the different sizes of the holes in the sieve-like membrane, and in order to decide this question, he examined the exchange of chlorine ions against sulfate ions, which are known to wander especially slowly.[19] Mond actually found considerable differences in the different animals. The interchange is quickest in the blood corpuscles of man, then there follow pig, horse, cattle. The conclusion that we have to come to in the experiment just described with differences in pore size has been supported by Mond by comparing the sulfate ion with the tetrahydric alcohol erythritol as a non-electrolyte which is insoluble in lipoids and which is known to penetrate into the blood corpuscles and other cells as slowly as the sulfate ion. The same result occurred, namely, the speed of permeation was greatest with the corpuscles of man and the least with those of cattle.

But there exist not only differences from animal to animal in the porous permeability of the cells; the same state of affairs holds for the solution permeability. It is well-known that almost every basic dye enters the living cell, but there are rather few acid dye-stuffs that are suitable to it. As to the sulfonic acid dyes, evidently only those enter which dissolve in the oil mixture worked out by Nirenstein and, as has been illustrated by a table in the beginning of my lecture, the dyes enter the cells the more as their relative solubility in the oil mixture is greater. Now I have discovered that the partition coefficient of blood corpuscles to surrounding solution differs under the same conditions from one species to the other; for example, the coefficient is greater with the blood corpuscles of the pig than with those of cattle and sheep, and with these greater than with those of the horse.[20] This is demonstrated for two dyes in Table V.

[19] Mond and Gertz, *Pflüger's Arch.*, **221**, 623, 1929.

[20] Unpublished experiments.

It appears at once that two explanations may be attempted: either we have to assume that the blood corpuscles of all four animals contain lipoids of the same quality, on which the dyes are distributed, but the quantity is greatest in the corpuscles of the pig and is smallest in the corpuscles of the horse; or we have to do with nearly the same quantity of the lipoids in every kind of corpuscle, but the lipoids differ qualitatively as to their power to dissolve dyes, the power being greatest with the pig and smallest with the horse. It is my opinion that we must prefer the second explanation; for whenever the passage of the dyes is dependent here upon the lipoid solubility,—and unquestionably this is the case,—then we must expect that a dyestuff penetrating into the blood corpuscles of the pig will also get through the corpuscles of the horse, even if their lipoid phase is very small; but I have found that, on the contrary, the corpuscles of the horse are nearly impermeable to several of the staining substances examined. Thus we conclude that not only the properties of the porous phase of the cell boundary, but also its dissolving properties, vary from animal to animal.

TABLE V

Partition of Dyestuffs to Blood Corpuscles

Kind of Corpuscle	Dye	Initial Concentration	Final Concentration	Partition Coefficient
Horse	Tropæolin 1	0.0025	0.0017	1.9
Cattle	Tropæolin 1	0.0025	0.0015	2.7
Pig	Tropæolin 1	0.0025	0.0013	3.7
Horse	Bromophenol blue	0.0025	0.0022	0.55
Cattle	Bromophenol blue	0.0025	0.0020	1.0
Pig	Bromophenol blue	0.0025	0.0015	2.7

Ladies and gentlemen, I have come to the end and I shall repeat now the previously quoted words of Professor Jacobs: "It may be emphasized that what is most needed in the field of cell permeability at the present day is facts." And in relation to that I wish to add to what I have already said to you a quotation from Professor Ralph Lillie's lecture concerning the scientific view of life. He said: "What is required is the imagination or construction of some model that will reproduce in intelligible form the essential features of the phenomenon under consideration. Intelligibility is the essential criterion of the scientific view; it aims at making phenomena intellectually comprehensible." We perceive better than anywhere else the striking advantage of using models in the development of our knowledge of cell permeability, beginning with Traube and Pfeffer and passing from

Overton to Collander and Michaelis. It is very peculiar that in this direction the physical chemists have realized almost nothing from these weary, but very fascinating and instructive studies of what is required as a model of the cell membrane. This is to their own disadvantage, I believe, because they here overlooked fundamental problems worthy of pursuit by the methods of exact science which are applicable to the membranes, the qualities of which have been discussed in this lecture. Under these circumstances the physiologist is constrained, and will be constrained still more in the future, to leave his proper work, as he must for a shorter or even for a longer time put away physiology, and become pure physicist or pure physical chemist in order to answer preliminary questions of great importance to physiology. Otherwise he will be open to the great danger of fabricating hypotheses. But whoever among the physiologists resolves to leave his physiological studies, he may encourage himself by remembering that it was Jacques Loeb who, feeling obliged to do so in regard to his science and to himself, created in the last years of his life a monumental work on the physical chemistry of the protein bodies. In this way he manifested anew his perseverance and his enthusiasm, both properties which distinguished also Reynold Spaeth, whose memory is with us today.

THE COPPER CONTENT AND THE MINIMAL MOLECULAR WEIGHT OF THE HEMOCYANINS OF *BUSYCON CANALICULATUM* AND OF *LOLIGO PEALEI*

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The view, originally put forward by Fredericq (1878), that copper is a normal constituent of hemocyanin and that it has a significance in the respiratory function of this protein similar to that of iron in hemoglobin has been substantiated by later investigations, particularly those of Begemann (1924) and Redfield, Coolidge and Montgomery (1928), which show that the combining ratio of copper to oxygen is the same in the blood of a large number of invertebrates. A knowledge of the quantity of copper in hemocyanin consequently provides significant information with regard to its respiratory function. Inasmuch as the amount of copper in the various hemocyanins does not appear to be the same, such data gives unequivocal evidence of the specific character of the respiratory pigments in the different groups of invertebrates. Furthermore, because of the very small number of copper atoms in the hemocyanin molecule, the copper content is a most valuable basis from which to estimate the minimal molecular weights of these proteins.

In this paper an investigation of the hemocyanin of the whelk, *Busycon canaliculatum*, and of the squid, *Loligo pealei*, is described. Mendel and Bradley (1906) studied the respiratory protein of the blood of the whelk, which they called hemosycotypin,—a name derived from the then current generic name of this form, *Sycotypus*. They report that it contained zinc as well as copper.[1] They concluded that copper composed only 0.043 per cent of the weight of the molecule, a value very much smaller than that obtained in the case of other hemocyanins and one which leads to very high estimates of the protein content of the blood when the oxygen capacities demonstrated by

[1] It seems preferable to include "hemosycotypin" among the hemocyanins because it has been demonstrated that the combining ratios of copper and oxygen are the same in this case as in that of other hemocyanins and because recent observations in this laboratory appear to make it doubtful whether the zinc is a true constituent of the protein molecule. Inasmuch as specific differences appear to exist between the hemocyanins of different groups of animals, confusion will be apt to result if each hemocyanin is given a different specific name.

Redfield, Coolidge and Montgomery (1928) are taken into account. The copper content of the hemocyanin of the squid does not appear to have been previously examined.

The copper content of these hemocyanins has been determined on material purified according to several standard procedures applicable to protein substances. Analyses for copper were made by the method described by Redfield, Coolidge and Shotts (1928). Between 10 and 20 c.c. of the hemocyanin solutions were used in each sample. The samples were dried in an oven at 100–110° C. for 48 hours, cooled in a dessicator and weighed. This procedure was repeated daily until successive weights did not vary more than 1 mgm. The samples of dried hemocyanin weighed between 100 and 300 mgm. Digestion, the electrolytic separation of copper, and its estimation were carried out exactly as described, except that in the titration 15 drops of potassium iodide were used instead of 10, as this modification was found to sharpen the end point.

We have not succeeded in producing definitely crystalline preparations of the hemocyanin of *Busycon canaliculatum* by methods which have been found applicable in other cases. Dhéré, Baumeler and Schneider (1929) have also been unsuccessful in crystallizing this hemocyanin. However, on prolonged dialysis against distilled water a precipitate is formed which appears to be composed of short rods and which gives a silky sheen on shaking similar to that characteristic of crystalline protein preparations.[2] *Busycon* hemocyanin appears to be a globulin, as it is insoluble in the region of its isoelectric point in salt solutions of sufficient dilution. This property has been used in purifying our material as well as the usual procedure of salting out with ammonium sulphate, employed by Redfield, Coolidge and Shotts (1928) in the preparation of *Limulus* hemocyanin.

[2] In an attempt to produce crystals, a number of preparations of hemocyanin, all of which showed a silky sheen on shaking, have been made by different methods from several species. The precipitated particles were too small, however, to be recognized under the microscope as definite crystals, though a very fine rod shape was observed in many cases. By the addition of 2 drops of serum to 1–2.5 c.c. of 0.05M acetate buffer solution of pH 4 to pH 5, the hemocyanins of *Busycon canaliculatum* and of *Busycon carica* were precipitated and showed a sheen on shaking. In the case of the bloods of the eight different species; *Limulus polyphemus* (horseshoe crab), *Busycon canaliculatum*, *Busycon carica*, *Libinia emarginata* (spider crab), *Loligo pealei*, *Homarus americanus* (lobster), *Callinectes sapidus* (blue crab), and *Ovalipes ocellatus* (lady crab), the hemocyanin was precipitated by diluting the serum 20 to 200 times and adding a few drops of 0.006 per cent acetic acid to 5 c.c. of the diluted serum. The acid must be added slowly or a precipitate will be formed which will show no sheen. Too much acid redissolves the precipitate.

In several cases these hemocyanin precipitates were concentrated by centrifuging and redissolved, whereupon the solutions appeared distinctly blue. This color disappeared when the solution was reduced with sodium hydrosulfite so that evidently the hemocyanin was not denatured by the process.

TABLE I

Copper Content of Hemocyanin of Busycon canaliculatum

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
IVa	Three washings at isoelectric point	0.2071	0.496	0.240
		0.2053	0.496	0.242
		0.2067	0.492	0.238
		0.2070	0.482	0.234
IVb	Four additional washings at isoelectric point	0.1541	0.366	0.237
		0.1538	0.378	0.245
		0.1544	0.371	0.240
		0.1554	0.378	0.243
VI	Three washings at isoelectric point	0.0914	0.225	0.246
		0.0919	0.204	(0.227)
		0.0916	0.217	0.238
		0.0917	0.230	0.238
		0.2694	0.642	0.238
		0.2699	0.634	0.235
		0.2698	0.633	0.235
		0.1816	0.437	0.241
		0.1821	0.433	0.239
VII	Salting out and dialysis	0.1694	0.440	0.260
		0.1680	0.436	0.260
		0.1700	0.441	0.260
		0.1699	0.443	0.260
		0.1693	0.436	0.258
		0.1693	0.438	0.258
		0.1693	0.434	0.256
		0.1693	0.440	0.260
		0.1693	0.441	0.260
		0.1693	0.434	0.256
VIII	Salting out and dialysis under conditions leading to precipitation	0.1075	0.263	0.242
		0.2130	0.517	0.242
		0.2120	0.530	0.250
X	Salting out and dialysis	0.3967	0.948	0.239
		0.3948	0.944	0.238
		0.3965	0.950	0.238
		0.3978	0.948	0.237
		0.6492	1.535	0.236
		0.6485	1.554	0.240
		0.6487	1.534	0.236
XI	Salting out and dialysis	0.5494	1.318	0.240
		0.5481	1.308	0.238
		0.5483	1.309	0.239
		0.5478	1.311	0.239
		0.5472	1.315	0.240

Specimen IVa was made from blood which had been preserved with toluene in the cold room for two weeks. It was diluted with ten times its volume with distilled water and brought into the region of its isoelectric point by the careful addition of 0.01N HCl. The precipitate resulting was separated by centrifuging and put into solution in the original volume of water by the addition of an amount of sodium hydroxide equivalent to the hydrochloric acid previously added. This process was twice repeated. The precipitate finally obtained was washed with distilled water. The final product contained only a trace of chloride. Whenever acid or alkali was added, it was run in through a glass tube which had been drawn to a fine point while the hemocyanin was being vigorously stirred. In order to determine whether further purification of this product could be obtained, the entire process of purification was repeated four more times on a portion of Specimen IVa, the resulting preparation being designated Specimen IVb. Specimen VI was made in a manner similar to Specimen IVa. Specimen VII was made from blood which had been preserved half-saturated with ammonium sulphate for a month. The precipitated hemocyanin was separated by centrifuging and dissolved in a large volume of 5 per cent saturated solution of ammonium sulphate. The solution was centrifuged in order that a small amount of insoluble material might be discarded, and the solution was reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was then dialyzed against 0.001N sodium hydroxide under 20 cm. Hg reduced pressure for two weeks, at the end of which time it was free of sulphate. The preparation of Specimen VIII included the same steps as Specimen VII, except that it was dialyzed against 0.001N sodium hydroxide for five weeks at atmospheric pressure. At the end of the fifth week a precipitate appeared in the solution which gave on shaking a silky sheen similar in appearance to that produced by protein crystals. The precipitate consisted of rod-shaped particles about 2 μ in length. The solution still contained traces of sulphate and was consequently centrifuged and the precipitate washed three times with a large volume of distilled water. The sulphate test was then negative. Specimens X and XI were prepared from material which had been kept over two years precipitated in half saturated ammonium sulphate. They were purified by reprecipitation with ammonium sulphate (pH 8.0), repeated three times, followed by dialysis against 0.0001 sodium hydroxide for 18 days. The preparation and analysis of Specimens X and XI were made by Miss Elizabeth Ingalls.

The results of the analyses of these preparations are given in

Table I. The copper content obtained in the case of preparations made in the various ways is very nearly the same. This fact may be taken as evidence that fairly pure preparations of the protein have been obtained. The fact that the copper content of Specimen IVb was not materially increased over that in Specimen IVa by additional washing is further evidence for the adequacy of the method of purification employed.

The best representative value of the copper content of *Busycon canaliculatum* hemocyanin appears to be 0.24 per cent. Specimen VII yields consistent values 0.02 per cent higher than this. Inasmuch as Specimens VIII, X and XI, prepared by the same general method, agree with the general series, it is probable that the high value obtained in the case of Specimen VII should be attributed to some systematic analytical error rather than to superiority in the method of preparation.

Two specimens, which were obtained by the dialysis of fresh blood without other attempt at purification, yielded a product which contained about 0.22 per cent copper. This material was free of chloride and had the same nitrogen content per unit weight as the others. The result would appear to indicate that another protein may be present in the blood, but that if so, it exists only in small amounts. In the case of *Limulus*, the hemocyanin appears to account for about 95 per cent of the protein of the serum. In order to investigate this possibility further an attempt has been made to determine how far the nitrogen content of the blood of *Busycon canaliculatum* may be accounted for by the hemocyanin contained in it as estimated from the quantity of copper present. The nitrogen content of Specimen X was determined by the Kjeldahl method. Successive analyses yielded 15.6; 15.5; 15.7; 15.5; 15.4; 15.7; mean 15.5 grams nitrogen per 100 grams dry weight. The copper content of Specimen X was 0.238 grams per 100 grams dry weight. One part of copper consequently corresponds to 65.2 parts of nitrogen. Two specimens of blood were analyzed for copper and nitrogen. The first contained 0.074 mgm. copper per c.c. and 4.92 mgm. nitrogen per c.c. From the copper content it may be estimated that it contained 4.84 mgm. nitrogen as hemocyanin. The second specimen of blood contained 0.066 mgm. copper per c.c. and 4.14 mgm. nitrogen per c.c. The hemocyanin concentration as estimated from the copper content would account for 4.3 mgm. nitrogen. It is evident from these measurements that hemocyanin will account approximately for all of the protein nitrogen in *Busycon* blood.

One preparation of the hemocyanin of the allied species, *Busycon*

carica, was made. The blood had been preserved in a precipitated condition in half-saturated ammonium sulphate for one year in the cold room. The hemocyanin was separated, purified by the procedure employed in the case of *Busycon canaliculatum* Specimen X. Analysis of the copper content of the purified material yielded the following values: 0.217, 0.235, 0.238 per cent. The copper content of the hemocyanin of this species appears to be approximately the same as that of *Busycon canaliculatum*.

The hemocyanin of the squid, *Loligo pealei*, may be readily crystallized by methods similar to those first employed by Henze (1901) in preparing crystalline *Octopus* hemocyanin, and consequently lends itself well to purification. Squid hemocyanin is insoluble in solutions containing high concentrations of ammonium sulphate. It was found that if enough saturated ammonium sulphate solution is added to the blood to form a very slight cloud of precipitated hemocyanin, a fuller precipitation in the form of crystals can then be produced by several procedures designed to decrease the solubility of the hemocyanin in the solution. These were: (1) the careful addition of increasing quantities of ammonium sulphate, (2) increasing the hydrogen ion concentration as in the Hopkins-Pinkus (1898) method of crystallizing albumen, or (3) raising the temperature. These methods can be used with success in combination. Crystallization by raising the temperature, which is presumably due to increasing the "salting out" effect of the ammonium sulphate at the higher temperature is particularly efficacious and has the advantage that it involves the addition of no reagents and may consequently be accomplished slowly so as to favor the formation of crystals. It was found that by raising the temperature from 0° C. to 30° C., a heavier crystalline precipitate is produced than by raising it to room temperature only. A temperature change within a range which will not denature the protein did not crystallize all the hemocyanin that was in the solution. Consequently, the yield may be increased by combining the temperature method with the addition of ammonium sulphate or of acid. When crystallization is produced in this manner, there is formed first a fine precipitate, visible under the microscope but apparently amorphous. This changes in a few minutes to fine rods and then to bundles of needles and finally to large needles. The process is much like that described in the case of *Eledone moschata* hemocyanin by Kobert (1903). The appearance of the crystalline rods is similar to that figured by Dhéré (1919, figure 4), in the case of the oxyhemocyanin of *Helix pomatia* formed in the presence of sodium sulphate. If large excess of reagents are added suddenly, the precipitate produced is

amorphous. Crystallization of squid hemocyanin was obtained more readily from fresh blood than from preparations which had been preserved in a precipitated condition in concentrated ammonium sulphate or from previously crystallized hemocyanin. Crystals which had been kept for a year in the cold room in their mother liquor (half saturated ammonium sulphate), were found to have become insoluble in distilled water. This phenomenon was observed by Craifaleanu (1919) in the case of crystals of the hemocyanin of *Octopus vulgaris*. Craifaleanu called this form "para-hemocyanin."

TABLE II

Copper Content of Hemocyanin of Loligo pealei

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
I	Salting out and dialysis	0.1485	0.384	0.258
		0.1486	0.371	0.250
		0.1502	0.388	0.257
		0.1490	0.376	0.252
II	Crystallization and dialysis	0.0785	0.194	0.244
		0.1620	0.386	0.238
		0.1624	0.390	0.242
V	Salting out and dialysis	0.4579	1.155	0.252
		0.4594	1.161	0.254
		0.4593	1.178	0.256
		0.4601	1.159	0.252
		0.4592	1.154	0.252

Analyses of the copper content of the hemocyanin of *Loligo pealei* have been made upon three preparations. Specimens I and V were prepared from blood which had been precipitated by the addition of ammonium sulphate to half saturation and kept in the cold room at about 5° C. for two years. The material had a fishy odor, which disappeared when it was shaken with air and from which the final preparations were entirely free. The precipitate was separated from the supernatant fluid with the centrifuge and was dissolved with a small volume of 5 per cent ammonium sulphate. The solution was again centrifuged to throw down any insoluble material, and the fluid was drawn off and reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was finally dialyzed until it was found to be free of sulphate. Specimen II was prepared by crystallization from fresh blood. The blood was chilled to 0°, and then sufficient saturated ammonium sulphate was added to

produce a very slight precipitation of hemocyanin. The temperature was then raised from 0° to 20°, when full precipitation was obtained. The precipitate was in the form of needle-shaped crystals about ten μ in length. The crystals were separated from the mother liquor by centrifuging and dissolved with 5 per cent saturated ammonium sulphate. Insoluble material was removed by centrifuging, and the hemocyanin was then reprecipitated as before. This second precipitate was not crystalline, however. The preparation was then dialyzed against water until free of ammonium sulphate. All three preparations had a clear blue-green color and became colorless in the characteristic way upon reduction with sodium hydro-sulphite.

Table II contains the data obtained from analyses of these preparations of squid hemocyanin, which all yield values for the quantity of copper in the molecule close to 0.25 per cent.

It is interesting to compare the values obtained for the copper content of the hemocyanin of *Busycon* and *Loligo* with those previously reported for other species, particularly with regard to their systematic relationships. In Table III are collected the various determinations

TABLE III

	Copper	Author
	per cent	
Cancer	0.32	Griffiths (1892).
Homarus	0.34	"
Sepia	0.34	"
Octopus vulgaris	0.38	Henze (1901).
Loligo pealei	0.25	
Helix pomatia	0.25	Burdel (1922).
" "	0.29	Begemann (1924).
Busycon canaliculatum	0.24	
Limulus polyphemus	0.173	Redfield, Coolidge and Shotts (1928).

of the copper content of hemocyanin which occur in the literature. It is noteworthy that the value obtained in the case of *Busycon canaliculatum* and *Busycon carica* does not differ greatly from those attributed to the other gastropod, *Helix pomatia*. The value obtained for *Helix pomatia* by Begemann, whose method of copper analysis we have employed, exceeds the value obtained with *Busycon* by an amount well in excess of the apparent experimental errors. These hemocyanins appear also to differ in certain other respects. *Busycon* hemocyanin cannot be crystallized by methods which succeed in the case of *Helix* (Dhéré, Baumeler and Schneider, 1929). *Busycon* hemocyanin is insoluble in the region of its isoelectric point in the presence of quite

appreciable amounts of salt. *Helix* hemocyanin, on the other hand, appears to be readily dissolved by very small concentrations of salt under these circumstances (Svedberg and Heyroth, 1929).

It is surprising that such a great difference exists between the copper content of the hemocyanin of the squid and that of the octopus. Inasmuch as the properties of the respiratory pigments in these two cephalopods appear to be very similar, we believe it to be desirable to redetermine these values by methods of preparation and analysis which are strictly comparable.

The weight of hemocyanin containing one atom of copper is given by dividing the atomic weight of copper, 63.57, by the fraction of the weight of hemocyanin due to this element. In the case of *Busycon canaliculatum* this fraction is 0.25×10^{-2}. The minimal molecular weight of *Busycon* hemocyanin thus appears to be approximately 26,500, when estimated upon the basis of its copper content. It has been shown, however, by Redfield, Coolidge and Montgomery (1928), that when hemocyanin becomes associated with oxygen to form oxyhemocyanin, one molecule of oxygen is combined with a quantity of hemocyanin containing two atoms of copper. Inasmuch as it appears highly unlikely that the oxygen molecule is dissociated into its constituent atoms in its reaction with the respiratory protein, it seems safe to assume that each molecule of oxyhemocyanin is combined with not less than one molecule of oxygen. The hemocyanin molecule must consequently contain at least two atoms of copper. Estimated on this basis, the minimal molecular weight of *Busycon* hemocyanin is approximately 53,000. In a similar way it may be calculated that the minimal molecular weight of the hemocyanin of *Loligo pealei*, estimated on the basis of its copper content, is 25,400, and when the oxygen-combining relations are taken into account, the combining weight appears to be approximately 51,000.

Summary

The hemocyanin of *Busycon canaliculatum* contains 0.24 per cent of copper and 15.8 per cent of nitrogen. Its minimal molecular weight is approximately 53,000.

The copper content of the hemocyanin of *Busycon carica* appears to be the same.

The hemocyanin of *Loligo pealei* contains 0.25 per cent of copper and has a minimal molecular weight of approximately 51,000.

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GROWTH AND DIFFERENTIATION OF THE COLONIES OF *ZOOTHAMNIUM ALTERNANS* (CLAP. AND LACHM.)

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Introduction

In a preceding publication (1922) I have insisted on the fact that colonial Vorticellidæ constitute an intermediary step between a population of like cells (cultures of free Infusoria) and a multicellular organism; unlike free cells with unlimited power of division, whose population growth theoretically follows a geometrical progression. The colonies of *Epistylis,* of *Carchesium,* or of *Zoothamnium* generally have a limited growth, following a special cycle, independent of a possible sexual cycle. In these colonies the lineage of each cell is perfectly defined by dichotomous ramifications of a common peduncle, and it is possible to show in a large number of cases the existence of somewhat differential divisions giving two sister cells whose power of multiplication is different. In certain species (*Epistylis arenicolæ, Epistylis Perrieri*) the first divisions can be dichotomous and equal, so that the mass growth of a number of individuals follows a geometrical progression; but soon the sister cells resulting from each division multiply unequally, and the growth approaches more or less an arithmetical progression.

On the other hand, the study of the growth of the common peduncle, which is considered as a product of the protoplasmic activity, shows that the latter may decrease in course of time. But the Vorticellidæ colonies form, from time to time, migrating individuals which may be of the same size as the other individuals (*Carchesium, Epistylis*) or more voluminous (some *Epistylis,* some *Zoothamnium,* called heteromorphic). In these individuals, and in these only, appear secretory granules already observed by Engelmann and more recently (1926) by Wesenberg-Lund, which seem to be connected with the formation of the peduncle, and one can consider the hypothesis of an active substance, or of a transformable substance, produced in a definite quantity and periodically, by certain individuals, which is divided among the descendants of the latter and at the same time is diminished little by little.

It appears then that the growth of a group of cells may be limited by

factors somewhat internal but altogether independent of the hypothetical notion of a " factor of senescence " [1]

The *Zoothamnium* called heteromorphic, about which I have given some detail in my paper of 1922, seems to give the most typical examples as to the rôle of these internal, or properly cellular factors, in the general form of growth of a colony and its limitation.

Claparède and Lachmann described in 1858 a marine species, *Zoothamnium alternans* (described later by Möbius under the name of *Z. Cienkowskii*); the aspect of the colonies, they say, is that of " un arbre à branches courtes et très régulièrement alternantes. La forme de ces familles a sa cause dans un arrêt de division spontanée qui frappe en général l'un des deux individus issus de chaque division. Lorsqu'un individu *A* se divise en deux individus *B* et *B'*, l'un des deux, *B* par exemple, ne se forme qu'un pédoncule fort court et son développement reste stationnaire à partir de ce moment, tandis que l'autre, *B'*, secrète un pédoncule plus long, puis se divise en deux nouveaux individus, *C* et *C'*, dont le premier, qui est toujours du côté de la branche opposée à celui où se trouvait l'individu *B*, ne forme qu'un pédoncule très court et ne se divise pas davantage tandis que *C'* forme un pédoncule plus long et se divise en deux individus *D* et *D'* et ainsi de suite."

That is not all; in *Z. alternans* and in *Z. arbuscula* Ehrb. or *Z. geniculatum* Ayrton (see Wesenberg-Lund, 1925, and Furssenko, 1925) the migrating individuals which will be the origin of new colonies and will thus begin a new cycle, are distinguished not only by a few morphological characters, but also by their voluminous size and the well-determined place where they originate in the colony, generally at the junction of the main branches. These large migrating individuals are the " ciliospores " of Wesenberg-Lund or " macrozoides " of Furssenko, much larger than the " trophozoides " or " microzoides " which constitute the most numerous individuals of the colony.

Ehrenberg had observed these individuals in *Z. arbuscula*, and had noticed that they result from the growth of an individual not unlike the others, but always situated at the junction of a branch. This author admits that one of the two individuals issued from a bipartition on the branch while the other grows without dividing, thus being, he says " the aunt " of the individuals of the branch. Claparède and Lachmann find this same condition in *Z. alternans*, but sometimes this growing indi-

[1] In other publications (1925–26) I tried to show that in several very different cases the idea of a factor of senescence could be replaced either by the hypothesis of differing speeds in a group of transformations necessary to cellular activity, or by the assumption of a " probability " of transformation which would be too long to develop here. (See Fauré-Fremiet and Laura Kaufman, 1928, and Fauré-Fremiet and H. Garrault, 1928.)

vidual may undergo a division. *Zoothamnium alternans* (Claparède and Lachmann) is found frequently on the coasts of Brittany; I have found it in abundance in Woods Hole and was able to follow the different stages of the colony cycle and of the formation of the "ciliospores." I observed a few phenomena of conjugation, quite sporadic, but I have not observed a sexual cycle analogous to the one discovered by Wesenberg-Lund in *Z. geniculatum* or described by Furssenko in *Z. arbuscula.*

Technic

In order to follow the complete evolution in a large number of colonies, I have used numbered slides, ruled in squares with a diamond point. These slides were first placed in a crystallization dish containing numerous colonies of *Z. alternans.* After several hours, they were removed and placed in a Petri dish containing sea water and examined under a binocular microscope. All individuals recently attached were carefully located and designated in numeral order; those whose peduncle had already developed or had already given the first division were removed with a needle.

After this operation, the slides were placed vertically on frames floating in an aquarium through which ran a strong current of sea water; this was done to avoid the deposit of particles and of microörganisms. The slides were then examined periodically and the different stages of the development of each colony were carefully recorded in function of time.

When the cytological examination of a colony is necessary, it is always easy to detach this colony with a fine pipette, in order to study it under the high power, *in vivo,* or after fixation.

The best technic for the study of the nuclear apparatus is the fixation by OsO_4 for a short time followed by boracic carmine stain. The presence (generally in the Vorticellidæ) of a cuticle and the contractability of a peduncle constitute two technical difficulties which are not easy to overcome; it may be necessary to cut the colony with a fine scalpel in order to isolate certain individuals which it is necessary to fix and stain.

Structure of the Colonies

The appearance of colonies of *Z. alternans* is very nearly that of a palm (Fig. 1); they have a main trunk and oblique branches placed alternately in the same plane, on right and left of the axis; the main trunk always bears at the top a terminal individual of rather large size; the lateral oblique branches bear a variable number of small individuals;

finally along the trunk, at the juncture of the lateral branches, are found the voluminous migrating individuals either macrozooids or macrospores.

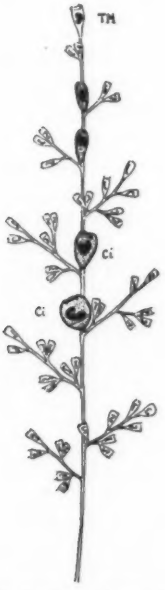


FIG. 1. A young colony of *Zoothamnium alternans* (Clap. and Lachm.), showing the main trunk and the alternate lateral branches. *TM*, terminal macrozooid; *Ci*, ciliospores at different stages of growth, located on the anterior side of the colony at the first division of each branch *D*, *E*, *G*, *H*. The branch *F*, in this case, bears, at the same place, only two microzooids apparently identical with the others.

The lateral branches of the colony observed in extension are almost always slightly curved in, and most of the individuals borne by these branches are inclined toward the outside of the curvature. The two sides of the palm are thus different, and one can define at the same time a base and a summit, an anterior and a posterior side.

The elements of symmetry of such a colony are a main axis represented by the trunk, and a median plane, antero-posterior, separating the two halves right and left.

As for all the other species of the genus *Zoothamnium*, the colonial peduncle bears an elastic tube whose rôle is passive, and a continuous "cordon central," dichotomically ramified, which represents the pro-

longation of the lower extremity of each individual; this central cordon has itself a protoplasmic tube (*I*) limited by a fine film and surrounding a muscular fiber which terminates at the basal part of each individual by a conical group of myonemes.[2] The migrating individuals, or "ciliospores," when liberated swim rapidly with their posterior ciliary crown. They are large individuals, flattened in the antero-posterior direction, and look like a top. They attach themselves by means of the scopula (*I*) and begin to secrete the peduncle. At the same time they lose their posterior ciliary crown and progressively take on again the ordinary subconical form.

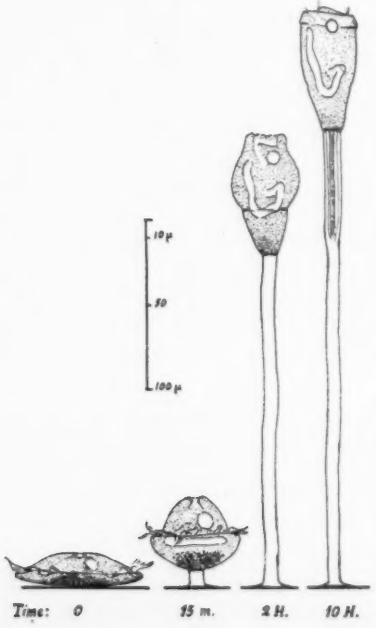


FIG. 2. Fixation of the ciliospore and construction of the peduncle. At first the top-like ciliospore turns quickly on the slide, then the building of the peduncle begins; the same individual is shown fifteen minutes after fixation. The ciliary crown slows down and disappears while the peduncle grows (Epistylis stage) during a short time (two hours); finally, one can see the differentiation of the "cordon central" and the muscular fiber (ten hours).

The peduncle is at first a solid cylindrical body of a fibrillar structure which grows rapidly ("Epistylis stage"); after two hours it reaches

[2] For the structure of the Vorticellidæ in general, and of the peduncle in particular, see Fauré-Fremiet (1906).

a length of about 250 μ. The secretion then begins to slow down and a section of the peduncle is ring-like; there is a central canal, at the bottom of which remains attached a part of the body of the infusorian, which from now on will lengthen itself along with the tube of the peduncle and become differentiated in a central cordon with the muscular fiber or "spasmoneme" (Fig. 2).

Six or seven hours (at the temperature of 21° C.) after the start of the secretion of the peduncle, the original individual undergoes a first unequal division which gives a macrozooid and a microzooid; the plane passing through these two zooids and the common peduncle is the median plane of symmetry of the future colony. The large cell remains clearly axial after this first division and continues to form actively the principal peduncle of the colony. After four to seven hours it undergoes a second unequal division; the interval between the following divisions is longer, from ten to sixteen hours; but always during the growth of the colony the terminal individual is a macrozooid, each division of which separates a microzooid in the median plane of the colony. The successive series of terminal microzooids constitutes a main strain perfectly schematized by the axial trunk of the colony.

We shall designate each cell of this series by a Roman numeral representing the division which started it; we shall have then the original individual, or ciliospore, then the series of macrozooids, I, II, III, . . . X, etc.

We shall designate with capital letters the corresponding series of median microzooids detached from the main strain (microzooids of first order), *A, B, C,* . . . *J,* etc. Each branch of the colony is started by the division, alternately at the right and at the left of the median plane of each microzooid of the first order. But, in accordance with the diagram of Claparède and Lachmann, only one of the two cells resulting from such a division is the origin of a lateral limb; we shall designate it by a small letter preceded by the coefficient 1; the other cell remains median and will be designated by its capital letter preceded by the same coefficient 1.

At the beginning of the formation of the fifth branch, for example, we shall have first the division of the terminal macrozooid IV, which will give a new terminal macrozooid V and a median microzooid *E*. The latter will divide in a perpendicular plane to that of the division of IV, and will give two individuals, one of which, 1*E*, remains in the median plane while the other, 1*e*, situated for example at the right of this plane, will be the origin of the branch (Fig. 3).

Each branch has also a main axis and lateral branches but does not have a well-defined median plane nor median individuals. The division

of $1e$, for instance, gives rise to two cells apparently similar, $2e^1$ and $2e^2$. The individual $2e^1$ remains in the axis and gives at the new division $3e^1$ (axial) and $3e^2$ (lateral); $3e^1$ will give $4e^1$ (axial) and $4e^2$ (lateral), etc.

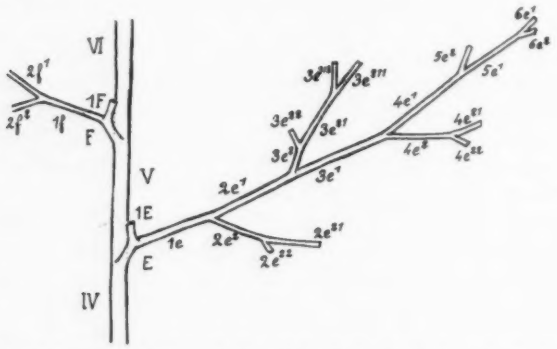


FIG. 3. Scheme of the branch E and the basis of the branch F, showing the lineage of the median microzooids $1E$ and $1F$ and the different microzooids.

Likewise the individuals $2e^2$, $3e^2$, and $4e^2$ will give successively two or three generations, the elements of which we shall designate by the symbols $2e^{21}$, $2e^{22}$, $3e^{21}$, $3e^{22}$, etc; according to the rule of Claparède and Lachmann $3e^{22}$ does not divide, but $3e^{21}$ gives $3e^{211}$ and $3e^{212}$; the number of generations formed by the lateral branches seems to be always rather limited.

The median individuals of the second generation: $1A$, $1B$, $1C$ . . . $1E$, etc., can divide once and give $1A^1$ and $1A^2$ for example. But while $1A$, $1B$, $1C$, and their two immediate descendants remain microzooids identical to these designated by the small letters, $1D$, $1E$ and the following ones, or the two cells of the second generation, $1D^1$, $1D^2$; $1E^1$, $1E^2$, etc., undergo a considerable growth and are transformed into ciliospores, or migrating macrozooids, which soon detach themselves from the common trunk to swim freely and to attach themselves later on.

It appears clearly then that during the growth of a colony of *Zoothamnium alternans* the two cells resulting from the division of one initial cell are never equivalent as to their "potentialities." But in confirming the observations of Ehrenberg and of Claparède and Lachmann, we may now make them more precise by showing that the progressive segregation of the power of multiplication and of the power of growth is very rigorously tied up with the respective position of the individual separated by the successive divisions. It seems then that a certain

number of divisions at least must be considered as differential divisions. The cytological examination confirms this interpretation.

First Division of the Initial Macrozooid

The first division is characterized, in a rigorously constant manner, by the unequal division of the macronucleus and of the protoplasm of the initial individual of the colony (Fig. 4) between the first two cells, the macrozooid *I* and the microzooid *A* (Fig. 5). A short time before this division, the macronucleus, which takes the shape of a long twisted rod, enlarges at one of its extremities in a compact mass. The other extremity is thin and often flattens slightly, and becomes elongated in the median plane of the individual. The two edges of this flat portion are often slightly thickened, so that a side view gives the impression of a structure in a horseshoe shape. The micronucleus remains near the thick extremity and soon lengthens into a spindle. Meanwhile the peristome and the scopula divide as well as the central cordon of the peduncle and soon an upper and a lower furrow, growing in depth toward each other, begin to separate two cells of very unequal size. The micronucleus completes its own division, then the macronucleus is divided unequally at the time when the two furrows join; the macrozooid (which remains the terminal individual on the axis of the colony) retains the thickened part of the macronucleus and a micronucleus; the microzooid (which becomes the first median individual *A*) retains the thin part of the macronucleus and a micronucleus (Fig. 6).

Considering the irregular shapes of the body and of the macronucleus in *Z. alternans,* it is impossible to calculate the corresponding volume and to establish the values of the nucleoplasmic relation. Nevertheless, it is clearly evident that the ratio N/P is greater in the microzooids than in the macrozooids, *i.e.,* the macronucleus is divided into two daughter cells even more unequally than the cytoplasm.

It is difficult to establish whether there exists a difference in composition between the two unequal extremities of the macronucleus divided between *I* and *A*. The "nuclear reaction" of Feulgen does not show any difference between these two parts, and their structure differs very little. Most frequently one can observe a linear orientation, in a continuous and parallel line of the chromatin granules (microsomes) in the thin part of the macronucleus which will be distributed by the division. On the other hand, the voluminous mass which remains in the macrozooid *I* shows an irregular distribution of its microsomes. This mass behaves as a chromatin reserve which would not be affected at all by the phenomena of division.

Supposing that the terminal condensation of the macronucleus repre-

sents a kind of segregation of the chromatin material, we shall describe this first unequal division as a differential quantitative and qualitative division.

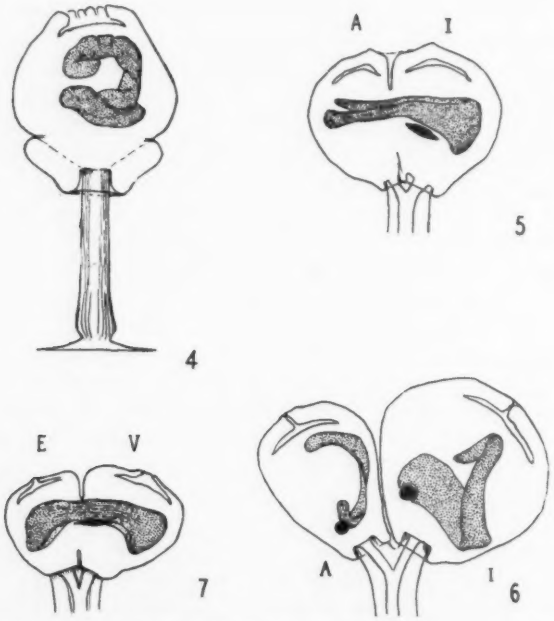


Fig. 4. Ciliospores at the beginning of the peduncle's formation, showing the appearance of the macronucleus before the first division.

Fig. 5. First cleavage of the ciliospore, giving the terminal macrozooid *I* and the median microzooid *A*. The figure shows the differential division of the macronucleus (figured by dotting) and the apparently equal division of the micronucleus (black—spindle stage).

Fig. 6. Later stage of the first cleavage, showing the terminal macrozooid *I* and the median microzooid *A;* macronucleus figured by dotting; resting micronucleus black.

Fig. 7. Fourth cleavage on the main strain giving the terminal macrozooid *V* and the median microzooid *E*. The qualitative equal division of the macronucleus (figured by dotting) is shown.

Later Divisions of the Individuals of the Main Strain

The division of the individuals *I* and *II* presents exactly the same differential character as that of the initial individual. It is different at the time of division of the individual *III*. In the latter, the macronucleus shows at the outset of the bipartition a symmetrical thickening at each of its granular extremities which appear entirely homologous.

The median part, finely striated, is divided, however, into two unequal parts by the division of the protoplasmic body, which isolates here again an axial and terminal macrozooid, V, and a median microzooid E (Fig. 7).

All the later divisions of the individuals from the main strain, *i.e.*, *IV, V, VI . . . X* etc., are of the same type, and we shall consider these divisions as quantitatively differential only.

Division of the Median Microzooids

The median microzooids, *A, B,* and *C,* which have received only the thin extremity of the initial macronucleus, undergo an almost equal division which gives for example $1A^1$ (median) and $1a^2$ (lateral) of the same dimension and of the same structure, both having a thin and twisted macronucleus, as well as the descendants of $1a^2$, $1b^2$, and $1c^2$ (Fig. 8).

On the other hand, the median microzooids, *D, E, F,* and the following undergo an unequal division, quantitatively and qualitatively differential, like that of the first three individuals: the ciliospores *I* and *II*. A short time before the division, when the median individual begins to lengthen in the transverse plane, its macronucleus takes the shape of an elliptic blade, presenting in a marginal point a large subspherical thickening. This thick part of the macronucleus, on the other hand, lengthens at the time of division and is divided between the two individuals 1*D*, 1*d*, 1*E* and 1*e*, etc. (Fig. 9).

These facts indicate that the differential division takes place at two different times from the fourth generation of the axial cells. For instance, when the division of *III* divides into *IV* and *D,* the microzooid *D* has a little less than a half macronucleus; but this half macronucleus is qualitatively similar to that of the macrozooid *IV*, having a granular terminal thickening. However, the microzooid *D* shows a nucleoplasmic relation, a ratio *N/P* superior to that of macrozooid *IV*, for the protoplasm has divided much more unequally than the macronucleus. It is a small individual with a large macronucleus.

When the microzooid *D* divides, the cytoplasmic division is almost equal, but the division of the macronucleus is qualitatively differential, because the thickened and granular part does not divide but goes whole to the median individual 1*D*. The outcome is that the ratio *N/P* is still increased in this individual.

The axial microzooids 1*D*, 1*E*, etc., can undergo a division and give for instance $1D^1$ and $1D^2$; but these two individuals, which remain median, soon begin to enlarge without dividing any further.

The microzooids 1*d*, 1*e*, etc., as said above, go through a series of divisions which always give individuals with long and slender macronuclei.

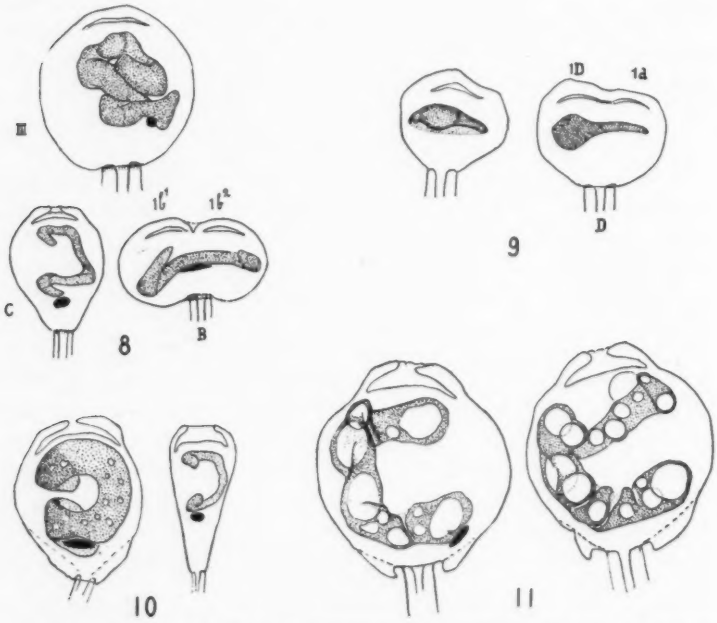


Fig. 8. Cleavage of the median microzooid *B*, giving, with equal division of the macronucleus, the microzooids 1b^1 and 1b^2. Comparison between the terminal macrozooid *III* and the median microzooid *C* (resting stage).

Fig. 9. Cleavage of the median microzooid *D*, giving the future median macrozooid 1*D* (ciliospore) and the microzooid 1*d*, with a differential division of the macronucleus.

Fig. 10. One median macrozooid (1*G* for example) at the beginning of its growth, and one microzooid of the corresponding branch. The large difference in size of the macronucleus is to be noted.

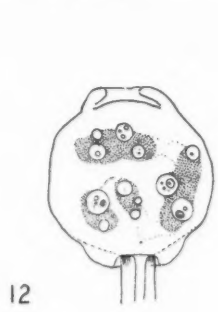
Fig. 11. Two median macrozooids during the time of growth. In the macronucleus, numerous large nucleoli are to be seen (figured as vesicles on the drawing).

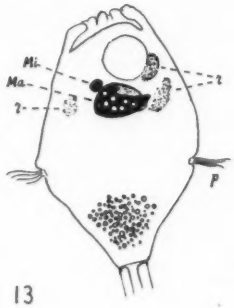
Growth of the Median Microzooids and Formation of the Ciliospores

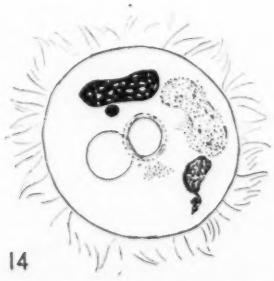
The median microzooids of the fourth generation (*D* or 1D^1 and 1D^2) and of the following generations (*E*, *F*, *G*, etc.) increase rapidly until they reach a length of about 55 μ to 70 μ, in one day, two days, or two and a half days.

The macronucleus, already voluminous, begins to grow and forms a very large horseshoe-shaped body. The micronucleus situated at the lower part in a slight depression lengthens into a spindle as in preparation for the division. While the macronucleus increases, rather refringent nucleoli appear in the midst of the chromatic granulations, not giving the reaction of Feulgen (Fig. 10).

Soon, while the protoplasmic growth goes on, it seems that the nuclear growth stops. The very numerous nucleoli alone still increase in volume (Fig. 11). Then the outline of the macronucleus disappears, the nucleoli project on the surface of the chromatic mass, and one can observe very numerous stages of disintegration and of degeneration of the macronucleus and of its fragments (Fig. 12).







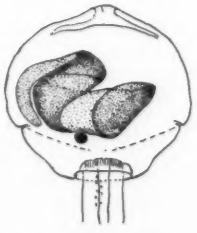


FIG. 12. Later stage of the median macrozooid's growth. Disintegration and disappearance of the macronucleus.

FIG. 13. One median macrozooid almost ready to leave the colony: *p*, posterior ciliary crown; *Ma* and *Mi*, macronucleus and micronucleus of the new nuclear apparatus; *r*, residual mass of chromatin.

FIG. 14. Top view of a median macrozooid (same stage as that shown in Fig. 12).

FIG. 15. Terminal macrozooid making the posterior ciliary crown and soon ready to leave the colony.

Finally, one sees in the center of the cytoplasmic mass containing a rather larger number of residual masses, a short macronucleus, arched, staining very intensely, containing only very small nucleoli, and accompanied by a resting spherical micronucleus (Figs. 13 and 14). This aspect, frequently observed, is that of a nuclear apparatus of new formation, and it is probable that the changes just described represent a phenomenon of endomixis. I was, however, unable to follow in the individuals stained *in toto* the fate of the spindle-shaped micronucleus observed in the preceding stages. It probably divides and makes up the new nuclear apparatus; but this stage was not observed in my set of preparations. At the end of the protoplasmic growth and when the nuclear changes are completed, a furrow appears around the median individual, at about the posterior third. It is the future ciliary crown, whose vibratile elements appear soon afterward. At the same time the organism flattens in the antero-posterior direction, and takes the shape of a top. The cytoplasm is filled with diverse inclusions, a great number of which are probably nuclear residue. In the posterior region, above the "scopula," appear very numerous inclusions which are not very refringent. Neutral red *in vivo* colors them a brownish red. These inclusions correspond to the secretion granules whose existence I have already mentioned in the migrating individuals of different Vorticellidæ.

There are still a few lipoid granules, and, toward the middle of the body, numerous small inclusions fixing neutral red in an intense red color. Iodine fixation gives a mahogany color, but the latter is not any stronger than for the microzooids.

The "ciliospore" which has thus been formed becomes almost lens-shaped. The peristome remains closed and the posterior ciliary fringe is animated with active movements which soon determine the liberation of the migrating individual (Fig. 15).

Growth of the Colonies of Zoothamnium alternans

At a temperature of 21° C., in an aquarium with running water, the growth of the colonies of *Z. alternans* goes on very regularly for a period of eight to ten days. Hence it is easy, by periodic examinations of a specific colony, to follow the increase in number of the individuals as a function of time. We have then a measure of the colony's growth. This measure is not very exact, because certain individuals grow without dividing and their mass is clearly larger than that of the others. However, the group of large cells given by the terminal macrozooid and the ciliospores is always rather restricted, and one can admit that the

appearance of the development is rather well represented by the variation in number of the individuals. A more important error may arise from the fact that some parasitic Infusoria (*Acineta*) very often get into the microzooids (especially the microzooid of the first branch) and multiply in this individual, which does not divide and soon falls off.

Because of this, it is necessary at every investigation to trace a total scheme of the colony studied, indicating the place of each individual, which with some practice, may be quickly made by examining the colonies in extension in a thin water layer with a low power objective. By this means it is possible to keep an account of the accidental influences; but when the number of individuals increases too much, beyond the eighth day, for example, this method of pointing becomes very difficult and soon impossible to use with precision.

TABLE I

Date	Time	Numbers of Colonies Examined											
		1	2	3	4	5	6	7	8	9	10	11	12
July 14	11 A.M.	0	0	0	0	0	0	0	0	0	0	0	0
" 15	11 A.M.	III	III	III	III	II	III	III	III	II	III	III	
" 16	12 M.	V	V	V	IV	IV	III	IV	IV	III	IV	IV	
" 17	4:30 P.M.	VII	VIII	VII	V	VI	V	VII	VI	V	VI		
" 18	9 A.M.	VIII	IX	IX	VII	VII	VII	IX	VIII		VI		
" 19	10 A.M.	IX	XII		VIII	VIII	VIII	XI	X				
" 20	11:30 A.M.	XI	XIV		XII	X	IX	XIII					
" 21	9 P.M.	XII	XVI		XIV			XV					
" 22	9 P.M.		XVII			XII							

Date	Time	Numbers of Colonies Examined										
		13	14	15	16	17	18	19	20	21	22	23
July 14	11 A.M.	0	0	0	0	0	0	0	0	0	0	0
" 15	11 A.M.	II	III	III		II	IV	I	III		II	III
" 16	12 M.	IV	V	V		III	V		V		IV	IV
" 17	4:30 P.M.	VI	VII			V	V		VII		VI	VI
" 18	9 A.M.	VIII	VIII			VII			IX		VII	
" 19	10 A.M.											
" 20	11:30 A.M.											
" 21	9 P.M.											
" 22	9 P.M.											

The simultaneous study of the growth of the various colonies placed in apparently identical conditions, on the same slide or on adjacent slides, shows at first that the speed of growth is not the same for all the colonies. We have already seen that the interval between two divisions varies in rather large proportion, in the same stage, in two different colonies (*i.e.* four hours to seven hours between the division of *I* and that of *II*; ten hours to sixteen hours between the division of *II* and that of *III*).

Table I shows the records of twenty-three colonies (experiment commenced on the 14th of July); the figure 0 indicates the initial macrozooid at the beginning of the peduncle formation, and the Roman numbers indicate the number of the terminal individual on the main strain; we see, thus, that on the fourth day, there may be a difference of two generations between different colonies and that on the eighth day the difference may be four generations. The whole number of individuals borne by each colony differs, of course, proportionally.

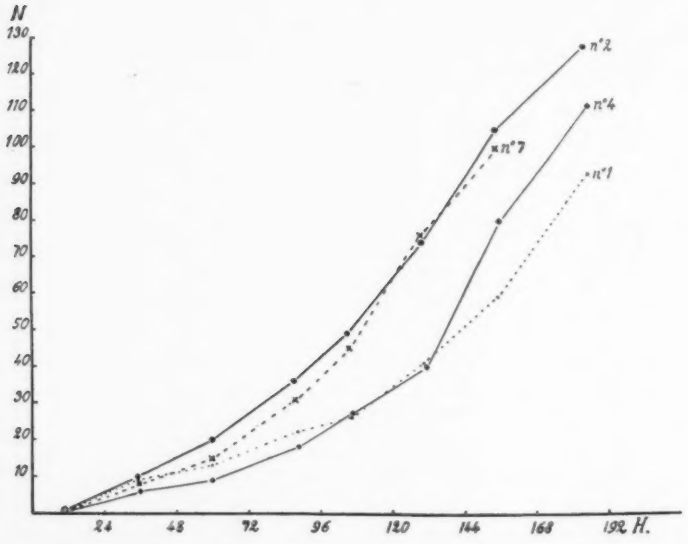


FIG. 16. Curves of growth from four colonies of *Z. alternans* (Nos. 1, 2, 4, and 7); number of the individuals in ordinates; time (in hours) in abscissæ.

The data relative to colonies Nos. 1, 2, 4, and 7 are plotted in the curve of Fig. 16. These are only gross numbers, there being no correction for some microzooids parasitised or dropped out. Besides, these various curves show that for each colony the rate of growth varies itself in the course of the growth; but it is difficult to determine the part of the accidental factors already mentioned and capable of introducing some disturbance.

Fig. 17 represents in function of time the genealogical and complete view of a colony having given sixteen generations on the main strain.

The essential data are given by the successive records of colony No. 2, completed, as regards the incomplete branches sprung from *A*, *B*, and *C*, by the data furnished by other colonies studied in the same experiment (3, 5, 20, etc.). Furthermore, the periods of some divisions have been settled according to the survey of the successive and periodical examinations of colony No. 2 with interpolations; I have kept account, in this case, of the interval settled with more precision than in other experiments in which either the first stages of the colony or the growth of a branch were connected at intervals of time most closely approached from hour to hour.

The curve represented in Fig. 18 is drawn according to this scheme. The daily increase of the number of individuals shows the following numbers:

Time (in hours)	Number of individuals	Increase of the unity of mass in 12 hours	Number of zooids made in 24 hours
0	1		1
12	2	2	
24	4	2	3
36	9	2.25	
48	15	1.66	11
60	23	1.58	
72	31	1.34	16
84	41	1.32	
96	55	1.34	24
108	66	1.20	
120	84	1.27	29
132	104	1.23	
144	122	1.17	38
156	137	1.12	
168	147	1.06	25

The first part of this tabulation shows a rather regular increase and such that the number of the individuals, *i.e.*, approximately the whole protoplasmic mass, doubles at regular intervals, from twelve hours to twelve hours.

Of course, we find again here, at first the geometrical progression of the ratio 2 which characterized the multiplication by bipartition of a mass of cells which keep always the same speed of growth. If we choose for unity of time this period of twelve hours, we see, however, that after the second day the rate of growth of the unity of mass, which averaged about 2, slows down progressively from 1.66 to 1.58 and 1.34, then persists for some time at a median and constant level: 1.32, 1.34, 1.20, 1.27.

Then, in a last period, this rate of growth again slows down with the

values 1.23, 1.17, 1.12, 1.06; but the difficulty in obtaining an exact enumeration does not permit a determination of its values when the colony approaches its greatest size.

Then it appears that the growth progressively slackens in the whole of the colony; *the time necessary to double the protoplasmic mass grows as the protoplasmic mass increases;* it is a limiting factor of the growth.

But it is evident that this factor (or limiting factor), in the case of *Z. alternans,* is not a factor of senescence which affects equally all the individuals, and involves a sort of progressive segregation, whose nuclear phenomena give a parallel objective picture.

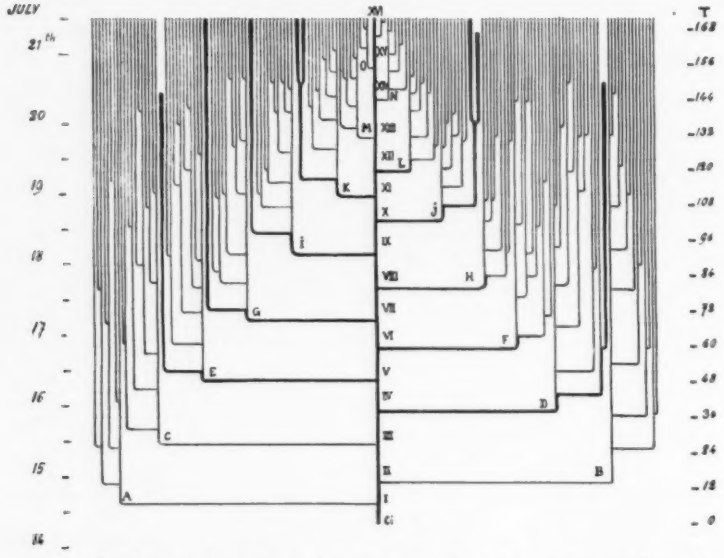


FIG. 17. Genealogical view of a colony at the sixteenth generation; time in abscissæ; lineage of each individual in ordinates.

This leads us to examine the case of the main strains. After the second division, which takes place rapidly, four to seven hours after the first one and at a temperature of 21° C., the rhythm of the bipartitions of the axial macrozooid slows down, (sixteen to seventeen hours between second and third divisions), then remains sensibly constant. During the entire growth of the colonies, more than twenty bipartitions of the axial macrozooid succeeded each other at intervals of ten to sixteen hours. The growth of the axial peduncle was fairly constant.

It seems then that during eight to ten days at least, the functional activity and the power of growth of the axial macrozooid remain constant, and, in the colonies already developed, one can observe the for-

mation of a posterior ciliary crown around the terminal individual. Thus the axial macrozooid can become a migrating individual equivalent to a ciliospore, but one never observes in this case the endomictic transformation of the nuclear apparatus. We have seen how the nuclear segregation which is established during the differential divisions seems to determine the characteristic features of the median individuals and of the microzooids. However, we must admit that the later divisions of the microzooids are still different, although they are not accompanied by a visible nuclear segregation.

According to the rule of Claparède and Lachmann, we can still distinguish in one branch one main strain and lateral strains.

The fourth branch, for instance, after the differential divisions which separate 1*D* and 1*d* may be represented as follows: 1*d* gives $2d^2$ and $2d^1$. Let us give the exponent 1 to the main strain of this branch; $2d^2$ gives $2d^{21}$ and $2d^{22}$ which do not divide any further; $2d^1$ on the contrary gives $3d^2$ and $3d^1$. The smaller branch issued from $3d^2$ has a principal axis, but the number of generations is reduced. The first division separates $3d^{22}$, which does not divide any further, and $3d^{21}$, which gives $3d^{212}$ and $3d^{211}$ without descendants. The individual $3d^1$ gives $4d^1$ and $4d^2$; $4d^2$ gives $4d^{22}$ without descendants and $4d^{21}$, which still gives $4d^{212}$ and $4d^{211}$ without descendants. The individual $4d^1$

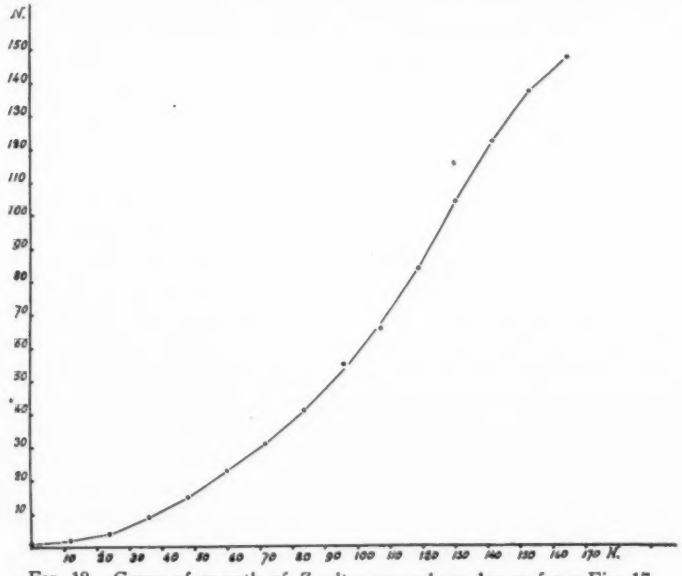


FIG. 18. Curve of growth of *Z. alternans* colony drawn from Fig. 17.

finally gives $5d^2$ without descendants, and $5d^1$ which divides into $6d^1$ and $6d^2$ without descendants.

The interval which separates the microzooid divisions is at first of the same order (or even more rapid) than the interval which separates the divisions of the axial individual; but it increases progressively and in such a colony, for example, the individuals of the sixth branch will represent six successive generations from the cell *F*, while its sister cell *VI* will have given during the same length of time ten successive generations.

We can see from Fig. 17, for instance, that the microzooids $2d^{22}$ and $2d^{21}$ live more than three days and a half without bipartition; such a fact is more typical with some microzooids of the earlier branches, *A* and *B*, which maintain themselves for more than five days without division. But after this time (corresponding to ten generations on the main strain of the branch) these individuals do not appear larger than the others; yet they feed and their protoplasm contains many digestive vacuoles. The decrease of the power of growth which characterizes these individuals is not dependent upon their age—and for this reason we cannot admit the notion of the factor of senescence—but of their position in the colony, as if the differential divisions assured the progressive segregation of a factor of growth. But we can still notice that this segregation, as it may be seen by the form of the growth of the branch *D*, for instance, is yet continued during the divisions of the microzooids which show no longer a differential appearance.

In short, if we bear in mind the main axis of the colony, its branches and its boughs, we see that the power of growth, and of multiplication, decreases according to a kind of gradient, in proportion with its removal from the main strain.

The differential character of the cellular divisions seems to be the essential condition which slows down and restrains the growth of the colonies of *Z. alternans*. But, theoretically at least, this restricted growth should go on indefinitely. It is not the case here. Secondary factors play here an important rôle; the development of different parasites (Protozoa, Protophytes) make it impossible to obtain a normal growth of the colonies beyond ten days, under ordinary laboratory conditions or in a natural marine environment; soon the last surviving individuals leave the common peduncle. The microzooids often form in this case a posterior ciliary wreath; their fate has not been determined.[3]

[3] A few cases of conjugation have been observed between a terminal macrozooid and a migrating microzooid. These cases were rare; the later phenomena were not followed.

Conclusions

The sexual cycle described by Furssenko and by Wesenberg-Lund in the voluminous species of *Zoothamnium* (*Z. arbuscula* Ehrb., *Z. geniculatum* Ayrton) is rather special and with *Z. alternans* (Clap. and Lach.) I have never observed anything similar, either on the Britanny coast or in my cultures at Woods Hole; I will not, then, attempt to compare the evolutionary cycle of these different species. The objective that has led me into the minute study of these colonies of Vorticellidæ is the cyclical evolution—generally considered—of an initial cell's lineage, which is here the foundation macrozooid or the "ciliospore."

The growth of colonies of *Z. alternans* is limited, in a great measure, by external agents such as parasitic infections, or the growth of animal and vegetable microörganisms which change the surrounding conditions of a specific colony.

In the cultures watched as described above, these various circumstances, somewhat accidental, are much reduced; yet the growth of each colony appears to be limited *in itself;* I have taken the common individual—the microzooid—as unity of mass, and I have observed that the rate of growth decreases in function of time for the whole of each colony studied; at the same time, some particular migrating individuals are formed and become the source of new colonies; it is precisely this "cyclical" appearance of growth in the colonies of Vorticellidæ that I have described in an earlier paper (Fauré-Fremiet, 1922); I have considered two different hypotheses: (1) the formation during the evolution of the migrating individuals of a limited stock of an hypothetical "active substance" which divides and becomes increasingly smaller with each generation of daughter-cells, or (2) a progressive modification of the intimate composition of the cells, variations which would be "corrected" only during the evolution of their own migrating cells.[4]

In any case, this cyclical and limited evolution gives to the colonies of Vorticellidæ (*Epistylis, Carchesium, Zoothamnium*) somewhat of an individualized character. In this regard, the case of *Z. alternans* is very striking. At first, the successive divisions of the cells derived from the first individual and the regularity with which they follow one another in exactly determinate planes which fix the general features of the colony, closely recall the process of a strictly predetermined cleavage, but one which would be complicated with a continuous growth.

Secondly, the existence in these colonies of a main strain and of secondary strains characterized by different nuclear qualities and different evolutionary properties recall in a certain measure the separation

[4] These suppositions have been examined and criticized in a very interesting work of G. Teissier (1928).

of the germinative and somatic strains during the cleavage of an *Ascaris* egg.

Thirdly and finally, we can characterize the individuality of the colony by the repartition of the power of growth and the power of multiplication of its cells according to a certain gradient.

In connection with another species of *Zoothamnium* Wesenberg-Lund also considers the notion of the individuality of the colony, for the various individuals are tied by the continuous protoplasmic thread of the ramified peduncle and this brings about in their mass rather a physiological unity. But the above-indicated characteristics are again met, more or less accentuated, in other colonial Vorticellidæ in the species *Epistylis* and *Carchesium*, for example, which do not show any protoplasmic connection between the zooids.

The case of these colonies is then nearer that of a " population " of cells, and their cyclical evolution appears very similar to populations of free Infusoria, studied by so many authors.

The case of *Z. alternans* is still, from this point of view, particularly interesting. In these species, the Claparède and Lachmann rule shows that two daughter-cells have not necessarily the same power of growth and of proliferation. I found the same rule (1922) in some species of the genera *Carchesium* and *Epistylis*, and more especially with *Epistylis arenicolæ* (n. sp).

Here there seemed to exist in the course of the successive bipartitions a kind of progressive segregation of the power of growth, but we find in *Z. alternans*, as an objective support of this hypothesis, the differential divisions, which are produced at the origin of each lateral branch and which indicate a kind of nuclear segregation.

In this species the main strain's cells which keep a constant nuclear appearance, keep also a constant rate of growth and, apparently, an indefinite multiplicative power. We witness, then, a cytological mechanism, probably independent of the external factors which rule the functional differentiation of the cells belonging to the same family, in a process of growth.

This cytological factor, or those which are superimposed upon it, rules at the same time the family's general mode of growth; it intervenes as a limiting factor, independent of the colony's age, and quite distinct, by this fact, from a factor of senescence in the true meaning of this word. However, the colony's initial individuals, the " ciliospores," appear to be characterized by a kind of " physiological potential " greater than that of the main strain's common individuals.

As in all the colonial Vorticellidæ that I have previously studied, they are characterized by large size and by the presence of definite

granulations connected with the secretion of the basic peduncle's inert substance.

During their particular growth, accompanied by a complete changing of the nuclear apparatus, the cells acquire these properties and we can thus show that near the end of the colony's cycle of growth an endomictic cycle exists, closely comparable to that observed in a population of free Infusoria.

But we must remark that, here again, the particular evolution of these "ciliospores" and the endomictic phenomena of which they are the seat, are determined, not by their *age*, but by their place in the colony's plan, just as if this evolution were still connected with the same mechanism of differential division and of nuclear segregation.[5]

I am very glad to be able here to express my thanks to the International Education Board, to my American colleagues who made my residence at Woods Hole so profitable for me, and, very particularly, to Dr. Calkins and Mrs. Harnley, who have helped me in translating this paper.

Summary

1. The first division of the initial macrozooid (or ciliospore) determines the median antero-posterior plane of the colony; the subsequent cleavages of the daughter individuals are brought about according to equally determined schemes, which give the main strain (or axial trunk) and the lateral branches, alternately at right and at left.

2. The individuals constituting the main strain are of a rather large size (axial macrozooids); their cleavage is always accompanied by a differential division giving rise to a new axial macrozooid and a median microzooid.

3. The differential divisions are characterized by an unequal division of the protoplasmic mass, accompanied either by a sensibly equal division of the macronucleus (division supposed to be quantitatively differential), or by the unequal division of the macronucleus in which the larger mass (delicately granular) remains in the larger individual, while the thinner part (often of fibrillar structure) goes to the microzooid (division supposed to be qualitatively differential).

4. The cleavages of the ciliospores and those of the axial macrozooids, I, II, and III are always differential as regards the protoplasm and the nucleus. The cleavages of the macrozooids IV and after give a cytoplasmic differential division and an equal nuclear division; the dif-

[5] Long ago I mentioned an apparently differential division in *Lagenophrys*, in which one of the individuals remained sedentary, while the other migrated and secreted a new shell (1904).

ferential division of the macronucleus is carried back to the cleavage of the corresponding median microzooids.

5. The common microzooids have a limited power of growth and of multiplication.

6. The median individuals having a large macronucleus after the differential division of the median microzooids *D* and progeny begin an active period of growth accompanied or unaccompanied by only one ulterior division: these forms constitute the median macrozooids or "ciliospores."

7. The growth of the ciliospores is accompanied by an important hypertrophy of the macronucleus followed at first by a disintegration, then by a reconstitution through an endomictic process.

8. During the growth of the median macrozooids, some grains of secretion accumulate at the individual's posterior end, then the ciliary crown grows, the ciliospore breaks away, swims freely, then settles down on a substratum and becomes the source of a new colony.

9. The character of the differential divisions on the main strain seems to determine the individual's differentiation of the colony; this differentiation depends not only on the individual's size, but also on its physiological potencies.

10. Independently of the obviously differential divisions, it is shown that the power of growth is divided among the microzooids according to a gradient, so to speak.

11. The unequal power of growth of the various individuals of a colony gives to its whole growth a behavior which approaches the behavior of an organism. This unequal share constitutes for the growth of the whole a limiting factor very unlike a factor of senescence.

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THE INFLUENCE OF HUMIDITY ON THE BODY TEMPERATURE OF CERTAIN POIKILOTHERMS

F. G. HALL AND R. W. ROOT

(From the Zoölogical Laboratory, Duke University)

Poikilothermic animals are commonly accredited with possession of a body temperature closely approximating that of their environment. In general this appears to be true. However, there are some cases where the body temperature of certain "cold blooded" animals may be very unlike that of their surroundings. Such examples are given by Rogers and Lewis (1916) in a table which they have compiled from the investigations of numerous workers. It shows that not all investigators agree even as to the temperature of the same species. It is probable that much of the discrepancy is due to different types of method. On the other hand, a more careful examination of the conflicting results of various authors as to the correspondence between body and environmental temperature shows that the greatest variations occur when animals are subjected to atmospheric conditions.

The factors which influence the temperature of animals may be classified as follows: *intrinsic*—those that lie within the organism and act to produce a temperature different from that of the environment; *extrinsic*—those imposed on the animal from without. The extrinsic factors are (1) conduction and convection, (2) radiation, (3) evaporation of water. A discussion of the rôle played by each factor is given by Pearse and Hall (1928). It is the purpose of this paper to study the influence of the third factor, namely, the evaporation of water, on the body temperature of various poikilotherms.

Experimental Methods

Apparatus.—The apparatus employed consisted of an air pump, several gas washing bottles—some containing concentrated sulfuric acid, others water—a chamber in which animals under experimentation were placed, temperature-measuring instruments, which included a potentiometer, a high sensitivity suspension galvanometer, and a copper-constantin thermocouple.

The air pump was adjusted to supply air at a constant rate of 22.6 liters per minute through two possible air leads. One lead was through

four wash bottles containing concentrated sulfuric acid and the other through four similar wash bottles containing pure water. The amount of air passing proportionally through each lead was controlled by screw pinch cocks. Thus air of any desired humidity from 7 per cent to 100 per cent could be obtained. The relative humidity of the air was measured by a calibrated hair hygrometer suspended in an enclosed jar through which all the air passed before entering the experimental animal chamber.

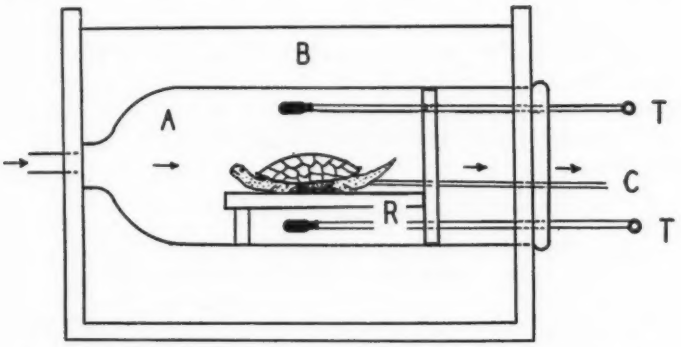

FIG. 1. Apparatus used to determine the influence of relative humidity on the body temperature of animals.

The experimental chamber in which animals were placed is shown in Fig. 1. A cylindrical percolator (*A*) was immersed in a constant temperature bath (*B*). Animals were tied to a sliding rack (*R*) which was so arranged that only a small portion of the animals' bodies was in contact with it, thus allowing a maximum surface to be exposed to the moving air. The end of this rack closed the mouth of the percolator. Two precision thermometers (*T*) were inserted through the rack, one in the upper, the other in the lower portion of the percolator. The thermocouple lead wires (*C*) also passed through the end of the rack. The mouth of the percolator was packed with cotton to lower the rate of conduction. The direction of air flow is shown by arrows. The temperature in all experiments was maintained at 20° C.

Experimental Animals.—The species chosen for this investigation were: Amphibians—the frog, *Rana pipiens* Schreber; the salamander, *Plethedon glutinosus* Green; the toad, *Bufo fowleri* Garman. Reptiles—the lizard, *Sceloporus undulatus* Latreille; the "horned toad," *Phrynosoma cornutum* Harlan; the turtles, *Terrapene carolina carolina* Linn., *Cistudo major* Agassiz, *Chrysemys marginata* Agassiz; the alli-

gator, *Alligator mississippiensis* Daudin. All animals were kept under good laboratory conditions, and were alive and active at the end of each experiment. Individuals were weighed at the beginning and end of each experiment. From four to ten individuals of each species were used and several determinations were made on each individual.

Temperature Records.—Environmental temperatures were recorded by use of precision thermometers placed in the experimental chamber. The body temperature was determined with a thermocouple inserted through the anus well up into the animal's body. Each thermocouple used was calibrated against a precision thermometer (previously calibrated by the U. S. Bureau of Standards). The temperature readings are believed to be accurate to $\pm$ 0.01° C. Records of the temperature of each animal and its environment were made at the following relative humidity points: 7 per cent, 25 per cent, 50 per cent, 75 per cent and 95–100 per cent.

TABLE I

Showing Variations in Body Temperature of Several Species of Poikilotherms from Environmental Temperatures in Atmospheres of Different Relative Humidities

Species	Relative Humidity				
	7	25	50	75	95–100
Salamander	−9.21	−6.34	−4.62	−2.54	−0.29
Frog	−8.60	−6.75	−4.68	−3.01	−0.13
Toad	−7.33	−5.31	−3.98	−2.48	−0.74
Lizard	−0.70	−0.70	−0.15	+0.30	+0.64
Horned "Toad"	−0.37	+0.02	+0.11	+0.19	+0.38
Turtle, water	−0.72	−0.57	−0.52	−0.41	−0.12
Turtle, land	−0.34	−0.23	−0.11	−0.03	+0.15
Alligator	−0.39	−0.26	−0.15	−0.08	+0.18

Plus signs signify a higher body temperature than that of the environment; minus signs indicate a depression in body temperature below that of the environment.

Results

Amphibians.—The body temperature of the salamander, frog, and toad very closely approximated that of their environment when the surrounding atmosphere was saturated, or nearly so, with water vapor. In atmospheres of low humidity, however, a considerable depression in the body temperature below that of the environment was obtained. Salamanders showed the most marked depression, toads the least marked. The average results obtained are shown in Table I. Con-

siderable weight loss was suffered by these animals. At low humidities their skins appeared dry and their bodies emaciated.

Reptiles.—The response of reptiles to atmospheres of varying humidity was quite unlike that of amphibians. Whereas amphibians showed great depression in body temperature when exposed to a dry environment, reptiles showed only slight depression. In fact, if the relative humidity be maintained between 90 and 100 per cent, many reptiles will show a body temperature slightly higher than that of their sur-

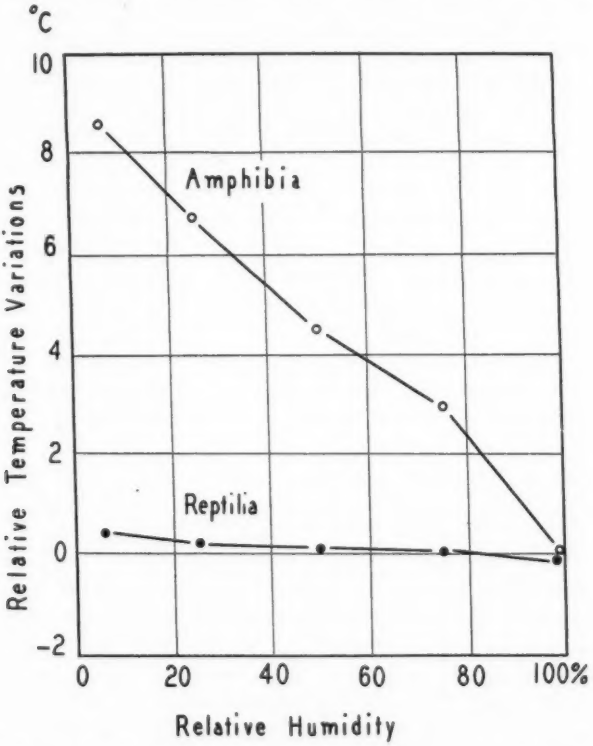

FIG. 2. Graph showing relation of body temperature to environmental temperature of amphibians and reptiles when subjected to different relative humidities.

roundings. Lizards and water turtles (*Chrysemys marginata*) were influenced the most by low humidity. Apparently the water turtle is slightly more susceptible to the influence of humidity than the land form. Weight loss in the reptiles was practically nil. Subjection to low hu-

midity for long periods of time showed no apparent injurious effect. Table I contains the average results obtained on all forms summarized to show the difference in response to surroundings of varying relative humidity. Fig. 2 shows the comparison of the response of amphibians as a group with that of reptiles, and shows the variation in the change of body temperature from that of the environment at similar relative humidities.

Discussion

It is apparent from the results obtained that in atmospheres of low relative humidity, amphibians will have a much lower body temperature than that of their environment. Such a condition results from the evaporation of water from the surface of the body. The body temperature of reptiles is but slightly changed by similar conditions. Thus it is clearly indicated that the difference in response of these two classes lies in the type of integument. The amphibians with moist skin will readily lose water by evaporation. They have little means of retaining water as has been shown by Gray (1928). The moisture of their integument is in dynamic equilibrium with the water content of their environment. The inner tissues supply water when that at the surface has been evaporated (Hall, 1922). Thus, for example, a salamander behaves physically very much like a wet bulb thermometer. The depression in temperature is not as great, probably because water is not transported to the surface as rapidly as in the wick of a wet bulb thermometer.

Amphibians are limited in their habitat to moist places. They possess a "reaction pattern" (Pearse, 1922), which permits them to live only under damp logs and stones or in marshes or other watery places. Thus they become more conspicuous on rainy days when the atmosphere offers a more favorable and less restricted environment for their activities. It is perhaps interesting to speculate that a frog may have a lower body temperature on a dry, sunny day than on a somewhat colder, rainy day.

The possession of a scaled integument, characteristic of the reptiles, greatly increases the power of water retention. Reptiles give up water very slowly and will resist desiccation for long periods of time (Hall, 1922). Not only by possession of an integument, but by certain internal physiological processes, such as the elimination of nitrogenous wastes as uric acid instead of urea, they conserve water. In consequence many reptiles live in very dry surroundings.

Perhaps the principal explanation of the discrepancies in reports by many investigators of the correspondence between body and en-

vironmental temperatures is that they are due to a lack of control or record of humidity. In the light of these experiments any results obtained without knowledge of the relative humidity of the surroundings in which an animal's temperature is taken would seem meaningless.

A further observation seems to indicate that the influence of changes in humidity on the body temperature of these animals decreases as animals higher in the phylogenetic series are used. It appears that

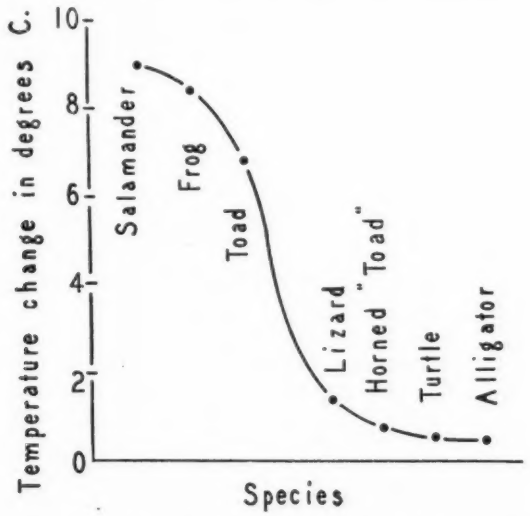

FIG. 3. Showing a comparison of the change in temperature of the body of each species studied when the humidity was lowered from 100 per cent to 7 per cent saturation.

amphibians as they progress in evolution show a decrease in their susceptibility to humidity variations. The same fact apparently holds for the reptiles. Fig. 3 represents the results arranged to show the maximum change in body temperature relative to environmental temperature in each of the species used, the salamander showing the greatest change, the alligator the least. The reptiles seem to have a more stable body temperature than amphibians because they are less influenced by environmental factors. Possibly the increased ability of water retention evolved in the reptiles is a " milestone " on the road to homoiothermism.

SUMMARY

1. Amphibians show marked response in body temperature to environmental variations in relative humidity. When subjected to an atmosphere of 7 per cent relative humidity at 20° C., a depression of several degrees centigrade may occur in their body temperature.

2. Reptiles show very little response to variations in relative humidity. The integument apparently prevents the evaporation of moisture from the surface of the body.

3. It is suggested that the evolution of the scaly integument of reptiles from the slimy and moist skin of amphibians, with the concomitant power of water retention, is perhaps an important step in the evolution of homoiothermism.

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THE POINT OF ENTRANCE OF THE SPERMATOZOÖN IN RELATION TO THE ORIENTATION OF THE EMBRYO IN EGGS WITH SPIRAL CLEAVAGE

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If the entrance of the spermatozoön into the egg is instrumental in determining the planes of cleavage, and the cleavage planes bear a definite relation to the embryonic axes, it would still remain important to find out whether the side of the egg on which the sperm enters is a factor in locating the dorsal (or ventral) side of the embryo. In some eggs having an equal first cleavage, such as the frog, the ascidian and the sea-urchin, observations of this kind have been reported, and a distinct relation has been found between the side of the egg on which the sperm enters and the future dorso-ventral axis of the embryo. Curiously enough, despite the large number of careful observations on the cell-lineage of eggs with a spiral type of cleavage, there is only one set of observations on the relation of the entering point to the first cleavage plane, and even here we do not know whether the side on which the sperm enters becomes the dorsal or the ventral side.

In the course of our work another relation was found that is both novel and has a bearing on the interpretation of the so-called law of alternate right- and left-cleavage in spiral types. In *Cumingia* it was discovered that two types of second cleavage occur in equal numbers, one of which in ordinary parlance would be called a right-handed, the other a left-handed spiral, yet in both cases the third cleavage was found to be always dexiotropic. As a consequence of this relation it follows that in one case the first plane of cleavage corresponds to the median plane of the embryo, and in the other case the second plane of cleavage corresponds to the median plane, provided the later sequence of events is the same for both types.

A third relation has not, so far as we know, been carefully studied, namely, whether in eggs with an unequal first cleavage, the plane of cleavage passes through the pole or consistently to the side. Without exception our observations show that the plane passes to the side on

which the smaller cell comes to lie, but the relations here are not the same in the three types examined, nor are the succeeding events always the same. However, these relations will be shown to have a significant bearing on the location of the median plane of the body.

The Cleavage of Cumingia

The early cleavage of the egg of the bivalve mollusk *Cumingia tellinoides* has been described by Morgan (1910) and Browne (1910). The following observations were made in the summer of 1929 at Woods Hole, Mass. The eggs and sperm were obtained by the usual method of isolating individuals in small dishes of sea water. The eggs were washed and samples removed for fertilization at once or soon after deposition. A square of vaseline was laid down on a slide and two fragments of No. 2 cover slips placed on the vaseline for additional support. A drop of eggs was placed in the square and a small drop of very dilute sperm-suspension was added. A cover slip was placed on the preparation and the slide was examined at once under the microscope. The eggs were brought under observation in less than thirty seconds after insemination. To some of the eggs one or more spermatozoa were already attached; to others they soon became attached. Only those cases in which one or a few spermatozoa were attached were followed—if the insemination had been too heavy the slide was rejected. The egg of *Cumingia* is about 66 micra in diameter, and with the jelly about 107 micra. The glass supports were about 140 micra in thickness, which with the further help of the vaseline sufficed to prevent compression of the eggs.

The pole of the egg of *Cumingia* can readily be identified by a clear area free from pigment. The outer pole of the first maturation spindle lies in the center of this area. The identification of the pole is later checked by the point of extrusion of the polar bodies. The sperm enters at any point of the periphery of the egg. On attaching itself to the egg the spermatozoön becomes immotile, its tail extending radially from the surface. About 30 seconds after attachment the egg rather suddenly becomes distinctly ovoid in shape, with the more pointed end at the point of attachment. This change in shape lasts 30 seconds or less. As the egg rounds out again the sperm enters. This phenomenon enables one to identify the particular sperm that will enter, even before the sperm-head has penetrated. Other sperms in the jelly, apparently even touching the surface of the egg, do not call forth this striking reaction. The change in shape is something more than the formation of a fertilization cone, since it involves a change in form of the whole egg. Unless the entering sperm is exactly on the horizon,

the change in form of the egg may not be observed; also there seems to be some difference in different sets of eggs as to its appearance.

The first polar body appears five or six minutes after fertilization. In making observations, all the sperms at or near the periphery of the egg were located on a drawing, and their relative position in three dimensions noted. Those that did not enter served as markers. Spermatozoa that are too far above or below the optical section of the egg cannot always be seen. When the polar body appears, the pole can be more accurately located in relation to the position of the entrance point. As a rule only one egg in each preparation was followed. The observations were made under magnifications of 284 and 440 diameters.

The first cleavage appeared about 50 minutes after fertilization. The location of the plane was noted in the drawing with respect to the point of entrance. This was checked as far as possible by the position of the markers, since, if any shifting of the egg occurred, their positions would change. The first division; Fig. 1, *a, b,* is unequal.

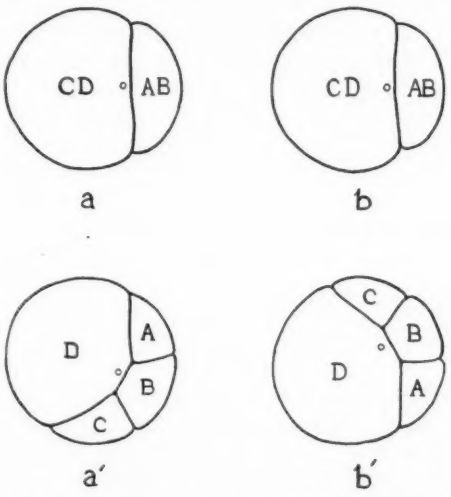

FIG. 1. The first and second cleavages of *Cumingia* showing the two possible types of 4-cell stage. In *a′* the *C*-cell comes off "counterclockwise"; in *b′* "clockwise."

The smaller blastomere, following the convention for this type of egg, will be called *AB*, and the larger blastomere *CD*. The second cleavage, Fig. 1, *a′*, *b′*, divides *AB* equally (*A* and *B*), and *CD* into unequal parts (*C* and *D*); the *C*-blastomere being smaller and approximately

the size of *A* or *B*. Theoretically the *C*-cell might form from either side of *CD* (Fig. 1, *a'*, *b'*). It is obvious, then, that there would be two possible configurations or arrangements of the blastomeres after the division that are mirror figures of each other (Fig. 1, *a'*, and Fig. 1, *b'*). As will be shown, it is important at this stage not to identify these two types as dextral or sinistral cleavages, although this would be the usual interpretation.

The clockwise sequence *ABCD* may seem to imply that the second cleavage has been leiotropic and the third will be dexiotropic, or conversely for the counter-clockwise sequence *DCBA*; but by utilizing the usual lettering we do not wish here to commit ourselves to such an implication. The reasons for this will appear later.

Entrance Point of Spermatozoön in Relation to the First Cleavage in Cumingia

Ninety-eight cases were recorded in which the relation of the entrance point to the first cleavage plane was definitely ascertained. In 77 cases there was strict coincidence between the plane of the first division and the entrance point. In 13 the entrance point was less than 45° from the cleavage plane. In 8 cases the divergence was greater than 45° and less than 90°. Whether the expectation of close coincidence should be 100 per cent and the departures be considered as due to abnormalities, or as due to errors of observation may be briefly considered.

Polyspermy might introduce a complication, but it can be detected either by the presence of extra pronuclei, or by irregularities in the cleavage. Compression of the egg might be one of the factors determining the position of the cleavage plane. To avoid this, the supports were made so thick that the space between the slide and the cover slip was greater than the diameter of the egg plus the jelly. If the sea water evaporates, the retreating edge of water may cause the egg to move, and the hypertonicity might cause irregularities in cleavage. This was avoided to a large extent by the wall of vaseline; also eggs were selected that lay in the centre of the drop. Any movement of the eggs can be detected by their position with respect to neighboring eggs. The change in shape that the egg undergoes before cleavage is not a serious source of error, especially if checked by the presence of "markers" on the egg, but during division the change in shape of the egg may cause slight changes in position. Therefore, whenever possible, the egg was constantly watched throughout this period. In some cases when the cleavage is horizontal the egg may roll over. This is prevented to some extent by avoiding jarring of the table etc. When

one or more of these factors was observed to come into play, the egg under observation was rejected.

The first cleavage plane does not pass through the pole (as determined by the position of the attached polar bodies), but slightly to one side. When considered from the entrance point of the sperm, the pole of the egg being up, this plane may be said to pass to the right or to the left of the pole. Whenever the first plane passes to the right of the pole, the *AB*-cell comes to lie to the right of the entrance point (Fig. 2, *a*); whenever it passes to the left of the pole, the *AB*

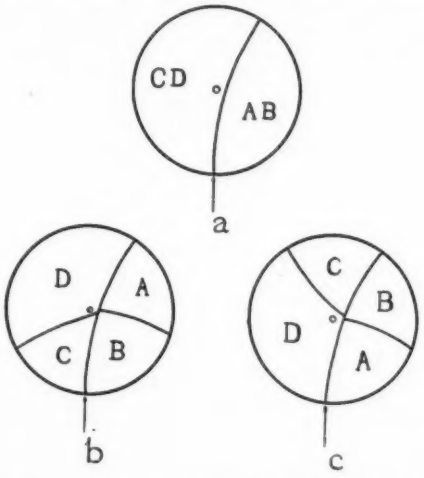

FIG. 2. The cleavage planes of *Cumingia* with respect to the entrance point of the spermatozoön. *a*, 2-cell stage with *AB* to right of sperm-entrance point; *b* and *c*, the two possible types of second cleavage.

comes to lie to the left (Fig. 3, *a*). This simple relation, which is constant in all the eggs examined, has apparently been overlooked by earlier observers in eggs of this type. The polar bodies adhere to the surface of the *CD* blastomere, and are carried into the furrow during the first division. Of the 77 cases of coincidence between the entrance point and the first plane, the *AB* was to the right in 40 cases and to the left in 37 cases. It appears that the chances are equal that the smaller cell lies to the right or to the left of the entrance point. The bearing of these two possibilities on the location of the plane of bilateral symmetry will be considered presently.

It is obvious that when the small cell (*AB*) lies to the right of the entrance point there are two possible types of second cleavage (Fig.

2, *b*, and Fig. 2, *c*); similarly when the small cell (*AB*) lies to the left (Figs. 3, *b*, and 3, *c*). As a matter of fact it was found in these 77 cases of coincidence that when the *AB* was to the right, only one of the two theoretical types appeared, namely, that shown in Fig. 2, *b*. When the *AB* was to the left, again only one of the two theoretical types appeared, namely, that shown in Fig. 3, *b*. Ordinarily the cleav-

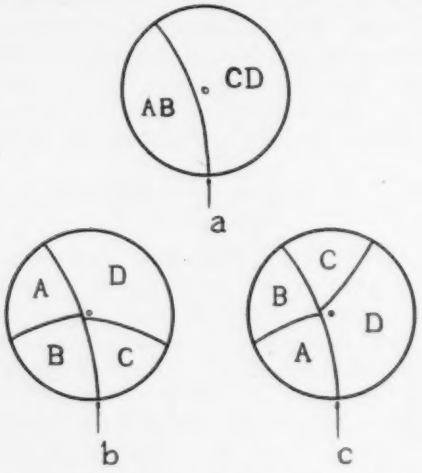

FIG. 3. The same as Fig. 2, except that the *AB*-cell lies to the left of the sperm-entrance point.

age giving the first type (Fig. 2, *b*) would be called a leiotropic second cleavage, implying that the third would be dexiotropic. The second type, Fig. 3, *b*, would be called a dexiotropic second cleavage, implying a leiotropic third. However, a study of the third cleavage of *Cumingia* has shown that the division is always dexiotropic. This information was obtained from eggs preserved at the time of the oncoming third cleavage. The orientation of the spindles with respect to the poles was determined in 84 eggs, and in every case they showed the cleavage to be dexiotropic (Fig. 5). The observation shows in the first place that it would have been erroneous to conclude that because the third cleavage is dexiotropic, the second must have been leiotropic. It would have been equally erroneous to have concluded from the two types of four-cell stages that the direction of the spiral would be different in the two types. By parity of reasoning it would seem unjustifiable to infer that because a given egg shows a leiotropic second cleavage, the first cleavage must have been dexiotropic, and thus to designate the egg as a dexiotropic egg.

Such reasoning might have led one to infer that a dexiotropic third cleavage in *Cumingia* means that the second cleavage must have been leiotropic. A study of preserved eggs in the anaphase of the second division gave no indication of a spiral arrangement of the spindle. The spindles in the *CD*- and *AB*-cells appear to lie in the same horizontal plane (Fig. 4, *a*, *b*, *c*, *d*), instead of being tilted in opposite directions, as has been described for other eggs at this division (Mead, Conklin). Of course it is possible that the tilting of the spindles in the *Cumingia* egg is too slight to be visible, but nevertheless it is in-

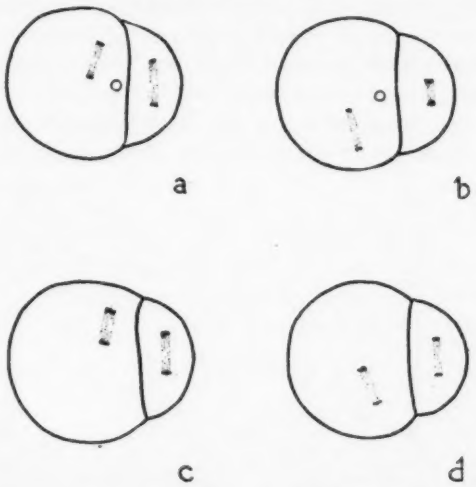

FIG. 4. Two-cell stages of *Cumingia* showing the positions of the spindle for the second cleavage. *a* and *b*, polar views; in *a* the *C*-cell will come off clockwise, in *b* the *C*-cell will come off counterclockwise. *c* and *d*, antipolar views; in *c* the *C*-cell will come off counterclockwise, in *d*, clockwise. The two poles of the spindles appear to lie at the same level in all cases.

teresting to note that in this egg in which two different types of four-cell stages occur the spindles do not show a visible tilting. The spindles, in the *AB*- and *CD*-cells, are horizontal as shown in the figures (Fig. 4, *a*, *b*, *c*, *d*). However, they are not parallel, but, especially in the *CD*-cell, the spindle makes an angle with the plane of division.

In order to answer the question, if it should arise, as to whether both types of cleavage in *Cumingia* produce normal embryos, a few eggs of each type were isolated. Normal embryos developed from each.

The normal trochophore swims in a dexiotropic spiral. This also occurred in the embryos from these two types. Moreover, *all* the em-

bryos from a culture swim in the same kind of spiral. In adult *Cumingia* the two valves of the shell are different in the articulation joint on the median dorsal side. All shells examined were alike, *i.e.*, not right or left, but all the same.

Location of the D-Cell in Relation to the Entrance Point

It has been found that when the first plane passes to the right of the pole (Fig. 2, *a*) the next division is always of such a sort that the *D*-cell is later away from the point of entrance of the sperm (Fig. 2, *b*). Similarly when the first plane passes to the left of the pole (Fig. 3, *a*) the next division is always of such a sort that the future *D*-cell is again away from the point of entrance (Fig. 3, *b*). The records from living eggs show that in 32 cases in which the cleavage plane passed to the right of the pole, the *D*-cell lay on the side opposite the entrance point, giving the arrangement of the blastomeres shown in Fig. 2, *b*. In 30

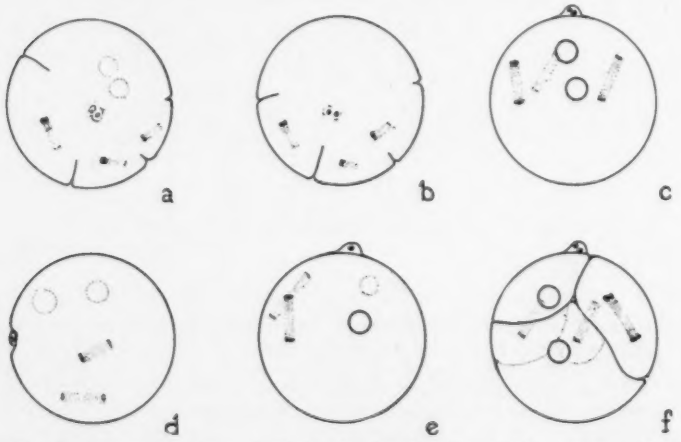

FIG. 5. Four-cell stages of *Cumingia* showing the position of the spindles for the next division. In all cases the spindles show that the next division will be dexiotropic.

cases in which the plane passed to the left, the *D*-cell also lay on the side opposite the entrance point, as in Fig. 3, *b*. No exceptions to this rule are found.

So far the description has been restricted to those cases where the first cleavage plane coincided very nearly with the entrance point. In addition there were a few other cases, as reported above, where the coincidence was not so close and where there were no reasons to sup-

pose that errors of observation were made. There were 13 such cases recorded in which the cleavage plane was less than 45° from the entrance point. If the entrance point is arbitrarily brought to the nearest point of the actual cleavage plane, then there are 3 cases in which *AB* is to the right of the entrance point, and 10 cases in which *AB* is to the left. The same relations of *D*-cell to entrance point obtain for both of these sets of cases as for those in which there was strict coincidence. There were also 8 cases in which the first cleavage plane was more than 45° from the entrance point. This divergence is too great to make a comparison profitable.

Relation of the Entrance Point of the Sperm to the Plane of Bilateral Symmetry

The evidence reported above has an important bearing on the relation of the point of entrance of the sperm to the plane of bilateral symmetry of the body. It has been shown that in 78 per cent of the cases close coincidence was observed between entrance point and first cleavage plane. In about half of these the first cleavage passed to the right of the pole (Fig. 2 *a*), giving the type of 4-cell stage shown in Fig. 2, *b*. At the next cleavage, the third, the 1-*d* micromere forms dexiotropically (Fig. 5). If from this point onwards the cleavages

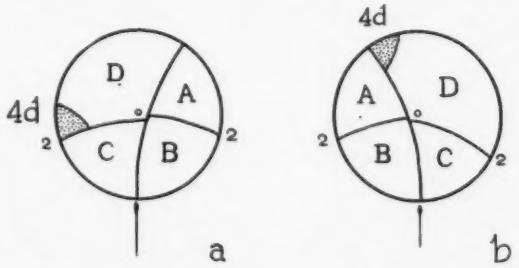

FIG. 6. Diagrams indicating the location of the 4-*d* cells in the two types of cleavage shown in Fig. 2, *b*, and in Fig. 3, *b*.

alternate, left and right, the 4-*d* cell will come off leiotropically and will lie next to the second plane of cleavage as shown in Fig. 6, *a*. It has been shown (Lillie, 1895) for at least one pelecypod (*Unio*) that the 4-*d* blastomere gives rise to the larval mesoblast, and establishes the plane of bilateral symmetry. This means that the second plane of cleavage coincides approximately with the median plane of the body.

In the other half of the recorded cases the first cleavage passed to the left of the pole (Fig. 3, *a*) giving the type of 4-cell stage shown

in Fig. 3, *b*. The 1-*d* again forms dexiotropically, Fig. 5. It follows from the same reasoning that the 4-*d* micromere comes off leiotropically, and will here lie next to the first plane of cleavage as shown in Fig. 6, *b*, and this plane of cleavage will now approximate the median plane of the body.

It may seem, then, that either the first or the second plane of cleavage may become the median plane of the body. This follows only on the assumption made above, which, although known to be true for other eggs, has not been entirely shown in this case. It is possible, for example, that the second somatoblast which determines the median plane may be formed at different divisions in the two cases. If, for example, in the type shown in Figs. 3, *a*, and 3, *b*, the second somatoblast appeared one division earlier or one division later, the median plane would be the same as in the other case (Fig. 2, *a*, and 2, *b*). As shown by the evidence, when the first cleavage plane passes to the right of the pole, the plane of bilateral symmetry coincides with the second cleavage plane, and when it passes to the left, with the first cleavage plane. What determines the passage of the first cleavage plane to the right of the pole in some cases and to the left in others is unknown. The fact that about 50 per cent of each type occurs suggests that it is merely a matter of chance. If we assume that the unfertilized egg has its materials radially arranged around the polar axis, and that the entering sperm determines through movements of the contents of the egg (or otherwise) that materials correlated with the determination of the *D*-cell come to lie opposite the entrance point of the sperm; and furthermore, that the cleavage plane does not pass through this material, then a possible interpretation suggests itself. It is obviously not necessary to make this assumption in quite the same crude form as suggested above in order to express these relations, for, at the time of the first division, all of the egg appears to be involved in the process. The risk of making such a generalization will be apparent when another egg, *Chætopterus,* is examined.

The Cleavage of Chætopterus

The eggs were washed in sea water, and allowed to stand about 20 to 30 minutes during which time the first polar spindle forms. A drop of eggs was put onto a slide prepared in the same way as for *Cumingia.* The egg measures 106 micra in diameter, without the jelly, and 111 micra with the jelly. The same thickness of cover slip support etc. was used as for *Cumingia.* A very small drop of very dilute sperm-suspension was added to the eggs which were examined immediately. In most cases the spermatozoa were already attached as though

the combination had been made almost instantaneously. The spermatozoön enters 15 to 30 seconds after insemination and may be missed unless the preparation is examined very quickly. The pole of the egg can be identified by the clear area in which the spindle for the first maturation division lies. The sperm enters at any point, and a slight fertilization cone appears at the point of entrance. The extra sperm which do not enter remain attached, and serve as markers. The exact position of the pole is given by the location of the polar bodies.

The cleavage of the egg of *Chætopterus* has been described by Mead, Wilson, and Lillie, and the relation of the median plane of the body to the first cleavage plane determined, but so far no one has examined the relation of the entrance point of the sperm to the first cleavage. The third cleavage of the egg is dexiotropic, and the fourth leiotropic, so that 2-*d* (the first somatoblast) comes off near the second cleavage plane, and 4-*d* (the second somatoblast) is similarly placed. This determines that the median plane of the body lies near the second cleavage plane.

The Relation of the Entrance Point of the Sperm to the First Cleavage Plane

As in *Cumingia* the location of the sperm that had entered was recorded on the drawing, and the individual eggs watched until the cleavage furrow appeared. In 48 eggs there was a fairly strict coincidence; in 35 eggs the entrance point was less than 45° from the plane of the first division, and both to the right and left of the plane. In 33 eggs it was more than 45° and less than 90° to the right and left. Thus in only 41 per cent of the cases was there a close agreement between entrance point and cleavage plane; but if the entrance point is not in some way correlated with the direction of the first cleavage plane, even this percentage of coincidence would not be expected. Taking first the cases where coincidence occurs, it was found that in 23 cases the first plane passed to the right of the pole, which means that the *AB*-cell lay to the right of the entrance point as in Fig. 7, *a*. In 25 cases it passed to the left of the pole, thus placing the *AB*-cell to the left of the entrance point as in Fig. 7, *b*. In both cases, however, the second cleavage gave the same arrangement of cells, namely, that shown in Figs. 7, *a'*, *b'*. According to the usual convention these four-cell stages would be obtained from leiotropic second cleavages (which is actually true for the *Chætopterus* egg), but in one type, Fig. 7, *a'*, the *D*-cell would lie away from the entrance point of the sperm, and in the other type near the entrance point (Fig. 7, *b'*).

The third cleavage in all cases observed, both in the living and in the preserved eggs, was dexiotropic. If the subsequent cleavages alternate left and right, the 4-*d* cell in both types will come to lie near to the second plane of cleavage (Fig. 7, *a'*, *b'*). This means that the second plane coincides with the median plane of the body, although in one type the entrance point of the sperm would be to the right of the median plane, and in the other it would be to the left.

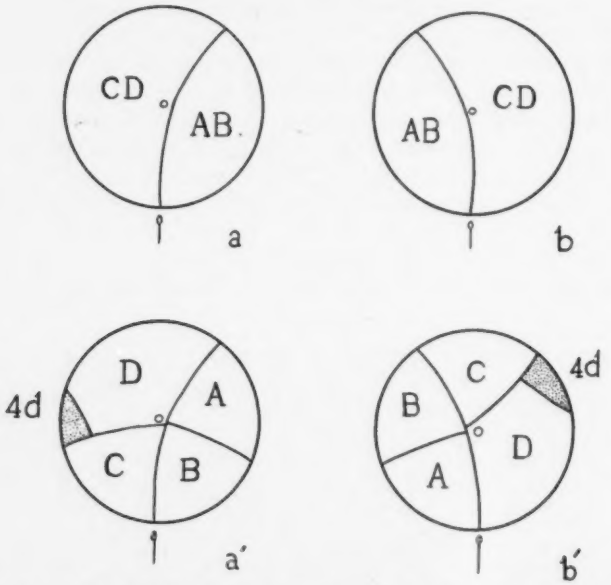

FIG. 7. Diagrams indicating the position of the first cleavage with respect to the polar body, and the entrance point of the spermatozoön; also the location of the 4-*d* cell. In *a'* the position of the 4-*d* resulting from the type of first division in *a* is shown, in *b'* that in *b*.

The Cleavage of Nereis

The egg of *Nereis* is particularly well suited for a study of relation of entrance point to cleavage, not only because the slow entrance of the sperm makes for accuracy of observation, but also because after the sperm-head has entered, a portion is left sticking to the fertilization membrane, and, if exactly on the horizon, may be still seen at the time when the cleavage begins. The technique was the same as for the *Cumingia* eggs, but since the egg is larger, thicker supports made from

glass tubing were used. Owing to the great thickness of the jelly a relatively large space between the cover and slide is essential. The location of the first cleavage with respect to the entrance point of the sperm has been studied by Just. The observations reported here were made to determine not only the constancy of the relation, but also to determine whether the *AB*-cell always forms to one side of the entrance point—a relation not previously reported. It was found that whereas the *AB* lay to the right in a very large number of cases, there were a few cases where it lay to the left. Nevertheless, at the four-cell stage only one arrangement of blastomeres was found (even in those with *AB* to the left), namely, that shown in Figs. 7, *a'*, or 7, *b'*.

The first plane of cleavage coincided with the entrance point in 33 cases. In 17 cases it was less than 45°. In 14 cases it was more than 45° and less than 90°. It is apparent from these observations that the agreement (51 per cent) is far from perfect.

Of the 33 cases of close coincidence, the first plane passed to the right of the pole in 28 cases, and in five cases to the left. Of the 17 cases less than 45° away, it passed to the right in 11 cases, and to the left in 6 cases. This conclusion was reached by arbitrarily shifting the entrance point to the nearest surface point in the cleavage plane. Here again there were more cases where *AB* lay to the right than to the left.

The configuration of the cells after the second cleavage is always of the same type (Fig. 7, *a'*, or 7, *b'*), whether the first cleavage passes to the left or to the right of the pole. In the 28 cases in which the first plane passed to the right, the *D*-cell formed away from the entrance point and in the five cases in which it passed to the left the *D*-cell formed near the entrance point.

The third cleavage of *Nereis,* as is well known, is always dexiotropic. The succeeding divisions of the egg alternate left and right. Hence, in both sets of cases the 4-*d* cell comes to lie near the second cleavage plane, which Wilson has shown to be near the median plane of the body.

In 1912 Just reported results of experiments on *Nereis* eggs, in which the entrance point was marked by the path of India ink in the jelly. He found coincidence varying from 50 per cent in one set to 60, to 80, to 95 per cent in other sets. He placed emphasis on those sets in which the greatest amount of agreement occurred. The exceptions he supposed were due to errors of technique, since by a change in technique he found in one set of 60 eggs, 100 per cent coincidence. Our own results gave only 51 per cent exact coincidence. That the vaseline we used was not injurious was shown by removing the eggs from the slide after the 4-cell stage and finding that they produced nor-

mal trochophores. We tried the India ink method in the hope of obtaining a large number of observations from a single preparation, but abandoned it because of the uncertainty in many cases of following the marker exactly to the surface of the membrane, and unless this can be done with absolute certainty there remains too great a chance of making a wrong inference, especially when the coincidence is not quite exact. In our opinion continuous observations on single eggs, while much more tedious, are safer.

Discussion

The main interest in these observations concerns the two types of the four-cell stages in *Cumingia*. As pointed out, one type arises in eggs in which the *AB*-cell forms to the right of the entrance point, and the other where it forms to the left. Since these two types give rise to two different planes of bilateral symmetry, *on the assumption made*, the problem of the determination of these planes seems to resolve itself into the problem of what determines that the cleavage plane lies to one or to the other side of the pole. Since these two types appear with equal frequency in *Cumingia*, it may seem that it is only a matter of "chance" to which side of the pole it passes. In *Nereis* there is only one type of four-cell stage and the *AB*-cell in the majority of cases (85 per cent) forms to the right of the entrance point. To this extent it conforms to the rule found for *Cumingia*. Since the *AB*-cell of *Nereis* lies to the right of the entrance point in 85 per cent of cases, its location does not here seem to be a matter of chance. In *Chætopterus* there is again only one type of four-cell stage, but here the *AB*-cell lies equally often to the right or to the left of the entrance point. Since there are here three different types of behavior leading to the formation of normal embryos, it may be inadvisable at present to try to reduce them all to one mechanism. The spiral type of cleavage common to all these eggs might incline one to attempt to find an explanation of the fact that the first cleavage plane passes to the right (with respect to entrance point) or to the left consistently in the different types. In *Cumingia* the egg regulates according to whether the *AB*-cell lies to the right or to the left of the entrance point. In *Chætopterus*, although the *AB*-cell again may lie either to the right or left of the pole there is no regulation, because the second cleavage plane coincides with the median plane. In *Nereis* no regulation is necessary, in this sense, in the majority of cases because these all conform to the same rule, but in the few exceptional cases the result is the same as in *Chætopterus*.

As already stated, an examination of the second cleavage spindle of *Cumingia* has not shown a spiral arrangement of the spindles. It

is equally obvious, however, that, just prior to the division, the spindle in the *CD*-cell lies well to one side, indicating the future position of the *C*-cell. After the division, the *A* and *C* blastomeres approach each other, more nearly in the polar than in the antipolar hemisphere in both types, while the *B* and *D* cells meet in a straight line at or near the antipole. If this be taken as evidence for a spiral second cleavage, then there are both leiotropic and dexiotropic second cleavages in *Cumingia*. Since the third cleavage is always dexiotropic this would contradict the "law" of alternating spiral cleavages.

It has been pointed out in the text that the two types of cleavage of *Cumingia* give rise to two different planes of bilateral symmetry. In one type the median plane coincides with this first cleavage plane, and in the other type with the second. This conclusion, however, is based on the assumptions that the law of alternating cleavage holds from the third cleavage on, and that the 4-*d* blastomere gives rise to the germ bands.

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THE EFFECT OF LOW OXYGEN TENSION ON THE PULSATIONS OF THE ISOLATED HOLOTHURIAN CLOACA

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The sequence of events in the respiration of *Stichopus moebii* Semper has been adequately set forth by Crozier (1916). In laboratory aquaria the rhythmic activity of the cloaca is distinctly periodic. A series of several pulsations is followed by a pause during which water is expelled from the respiratory tree. Then another series of inspirations begins. The number of inspirations in a series was found by Crozier (1916) to range from five to eleven, the greatest number being found in the largest animal. Pearse (1908) pointed out that, if the respiratory pulsations of *Thyone briareus* are prevented for some time by repeated mechanical stimulations, the contractions which ensue when stimulation ceases are greatly augmented in amplitude.

Oxygen deficiency has often been associated with periodicity and augmentation of response in various tissues. Douglas and Haldane (1909) have described periodic breathing in man under low oxygen tensions, and Douglas (1910) found the same type of breathing at high altitudes. Magnus (1904) and Frey (1923) reported that a stoppage of the oxygen supply to beating smooth muscle results immediately in an increase in amplitude. The present paper deals with the phenomena which have been observed on decreasing the oxygen available to a rhythmically beating isolated strip of circular muscle from the cloaca of *Stichopus moebii* Semper. This holothurian is found in great numbers in the shore waters at the Bermuda Biological Station. During the summer of 1927 the author repeated some of the experiments on a ring preparation from the cloaca of *Cucumaria frondosa,* very abundant at the Mount Desert Island Biological Laboratory, Maine.

Method

Crozier (1916) has shown that the cloaca *in situ* in the isolated posterior end of *Stichopus* will maintain its pulsations for many hours. No reference to the use of an isolated strip of this organ could be

[1] Contribution number 158.

found in the literature. The present work was carried out with an opened ring of the circular muscle of the cloaca. A cloacal-end preparation was first made similar to that described by Crozier (1916). The cloaca was then excised by cutting the radial muscles with a scalpel and freeing the organ from the anal rim by a transverse cut. From the muscular tube thus obtained a strip was made, one to two centimeters broad, and from four to six centimeters long. This strip was suspended vertically in a vessel of sea water by means of an L-shaped glass rod and a counterbalanced aluminum lever. A 250 cc. graduated cylinder cut off to hold about 125 cc. was found convenient as a vessel to hold measured amounts of sea water, or through which sea water could be made to flow continuously. The temperature of the water was recorded and found to vary little during an experiment, or from day to day. Therefore no special precautions for maintaining constant temperature were necessary.

Results

Records were taken from strips of *Stichopus* cloaca beating under the following conditions: (1) in a continuous flow of sea water, (2) in a limited amount of sea water, (3) in boiled sea water with added carbon dioxide, (4) in boiled sea water of various degrees of aeration, and (5) in normal sea water with potassium cyanide added.

Continuous Flow of Sea Water.—When sea water was made to flow continuously through the vessel at the rate of about 100 cc. a minute, the strip beating therein gave a tracing which was exceedingly uniform over a period of several hours, as may be seen in Fig. 1. Both amplitude and tone increased during the first hour. This condition was maintained for an hour or more. Then the tone began to fall very gradually while the amplitude remained about the same. After five to seven hours from the beginning, the amplitude began to decrease slightly. The rhythm was exceedingly regular and no indications of periodicity appeared. The rate of beat decreased slowly from the start, in one case almost 50 per cent after seven hours and forty-one minutes; but the preparation was still vigorous and regular.

Limited Amount of Sea Water.—When a strip was allowed to beat in a limited amount of sea water, that is in 100 cc. without change, the amplitude began to increase in about three hours and distinct periodicity developed as seen in Fig. 2. The increase in amplitude continued for an hour or more, becoming 230 per cent in one case. The tone was maintained until the increase in amplitude occurred, when it gradually fell; but the increase in amplitude was not entirely due to a decrease in tone since the contractions of the strip raised the lever a greater

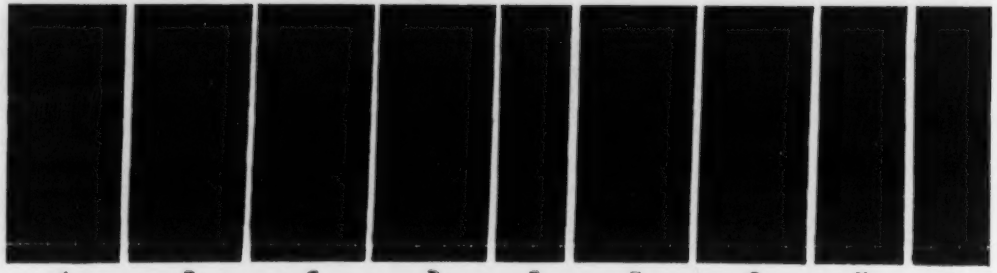

FIG. 1. Experiment 47. An isolated cloacal strip from *Stichopus mœbii* beating in sea water flowing continuously through the vessel. In this and subsequent tracings the large divisions on the base line mark time in minutes. Time after immersion of strip: *A*, 12 minutes; *B*, 45 minutes; *C*, 81 minutes; *D*, 139 minutes; *E*, 210 minutes; *F*, 300 minutes; *G*, 345 minutes; *H*, 390 minutes; *I*, 435 minutes. Duration of experiment 7 hours and 45 minutes.

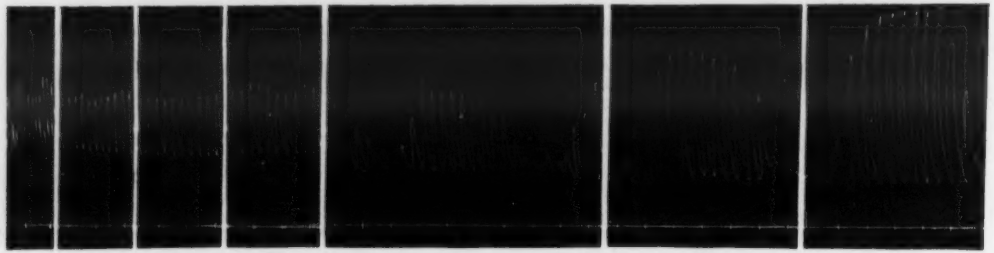

FIG. 2. Experiment 44. Cloacal strip beating in 100 cc. of sea water. Time after immersion of strip: *A*, 1 minute; *B*, 28 minutes; *C*, 60 minutes; *D*, 121 minutes; *E*, 161 minutes; *F*, 185 minutes; *G*, 222 minutes. Duration of experiment 3 hours and 48 minutes.

distance above the base line than in the beginning. Finally both amplitude and tone fell markedly. The rate of beat decreased constantly from five or six at the beginning to two or three per minute during the periods of beating. The length of the periods of inhibition of beat gradually increased to three or four minutes.

Boiled Sea Water.—Sea water which had been boiled in a narrow-necked flask and cooled to laboratory sea water temperature (28° C. or 29° C.) was used. When the muscle strip was immersed in 100 cc. of this water, the first two or three beats usually increased in amplitude, but both tone and amplitude almost immediately fell and the strip ceased beating in from three to five minutes as shown in Fig. 3, *A*. If the

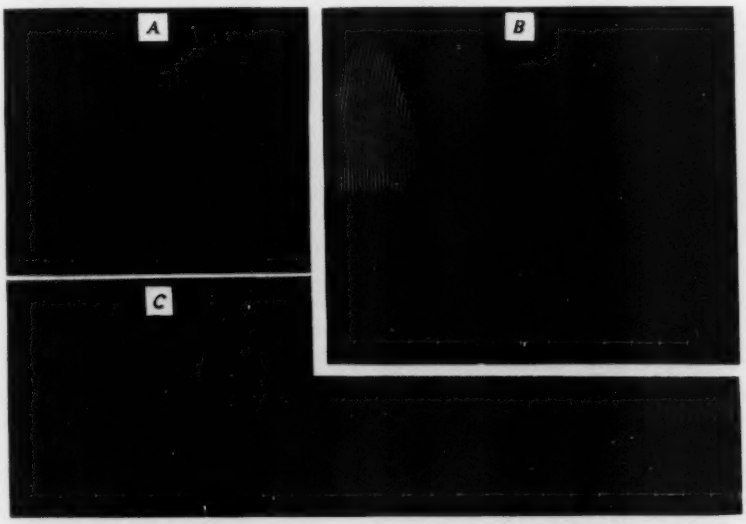

FIG. 3. *A*. Cloacal strip in 100 cc. of boiled sea water, pH 8.4. *B*. Cloacal strip in 100 cc. of boiled sea water treated with carbon dioxide, pH 5.8. Aeration at *X*. *C*. Cloacal strip in 100 cc. of boiled sea water, pH 8.3. Aeration at *X*. pH 8.2 immediately after aeration. pH 8.2 after 102 minutes.

water was aerated within three minutes by sucking it into a hypodermic syringe and squirting it back forcibly, a partial recovery occurred, which showed periodicity at first but later an uninterrupted rhythm (see Fig. 3, *C*). Several attempts to bring about recovery after waiting a longer period failed. The pH of the boiled sea water (indicator method) was sometimes as high as 8.8 as compared with 8.1 to 8.3, the pH for unboiled sea water in this region.

Boiled Sea Water with Added Carbon Dioxide.—Inasmuch as boiling removed the carbon dioxide as well as the oxygen, the former was replaced by means of a carbon dioxide generator. This resulted in boiled sea water ranging from pH 5.8 to 7.7. At the latter value the strip ceased to beat in three and one-half minutes and at the former value cessation occurred in three minutes. Aeration of the water after a three minute period of cessation failed to induce recovery (see Fig. 3, *B*). It seems therefore that neither the lack of carbon dioxide in the boiled sea water nor the increased alkalinity was the cause of the cessation of the pulsations.

A moderate excess of carbon dioxide was produced by treating 125 cc. of unboiled sea water with carbon dioxide until the pH was 7.0. This procedure was brief and probably did not remove much oxygen. In experiment 75 (Fig. 4) the amplitude began to decrease slowly after an hour, the rate decreasing gradually from the beginning. Neither augmentation of amplitude nor periodicity had appeared when the experiment was stopped after two hours and fifty-six minutes. When, however, an excess of carbon dioxide was produced by adding a few drops of N/10 HCl to a preparation beating in 100 cc. of unboiled sea water, there was an immediate rise in tone and increase in amplitude which soon gave way to a fall of tone and amplitude and finally to cessation of beat. It is therefore not probable that an accumulation of carbon dioxide in the immersion fluid as a result of tissue activity in a limited volume of water is the cause of the appearance of periodicity although it might be called upon to account for the increase in amplitude.

Boiled Sea Water of Various Degrees of Aeration.—When a decreased oxygen content of the sea water was produced, either by mixing boiled sea water with unboiled sea water or by partial aeration of boiled sea water, the augmentation and periodicity appeared much sooner than when a limited volume of unaltered sea water was used, the onset varying from a few minutes to two hours, according to the degree of oxygen lack. In one case the boiled sea water had been stored for several hours in a narrow-necked flask with only a few square centimeters of water surface exposed to the air. A strip beating in 100 cc. of this water became periodic at once and each successive period showed an increase in amplitude which finally amounted to about 200 per cent. The tone and the rate of beat, however, fell rapidly.

In another experiment in which 100 cc. of boiled sea water had been partially aerated, wave-like variations in amplitude appeared 13 minutes after immersion, and gradually developed into periodicity 53 minutes after the start. The amplitude increased from 10 mm. to 24

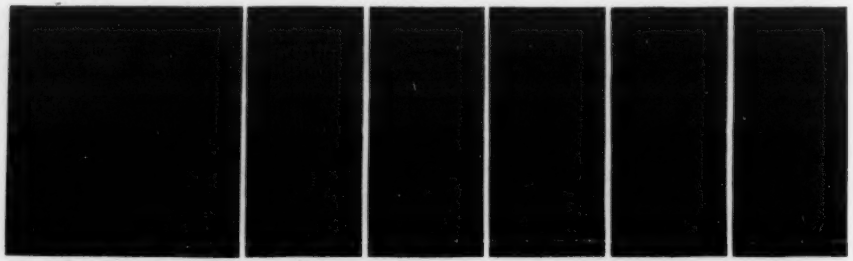

FIG. 4. Experiment 75. Cloacal strip in 125 cc. of sea water treated with carbon dioxide, pH 7.0. *A*, beginning at 10:00; *B*, 10:30; *C*, 11:00; *D*, 11:30; *E*, 12:00; *F*, 12:52. At the end, 12:56, pH 7.0.

FIG. 5. Experiment 62. Cloacal strip in 100 cc. of boiled and partially aerated sea water. Before boiling, pH 8.3. After boiling and partial aeration, pH 8.4. Time after immersion: *A*, 5 minutes; *B*, 32 minutes; *C*, 97 minutes; *D*, 117 minutes; *E*, 181 minutes. pH 8.4 after 166 minutes.

mm. and was still high when the experiment was stopped at the end of 93 minutes. The tone fell rapidly during the first five minutes and then more slowly during the next ten minutes after which it was unchanged. The rate decreased about fifty per cent during the first half hour and then remained constant.

In experiment 62 (Fig. 5) the boiled sea water (100 cc.) was partially aerated. Before boiling the pH was 8.3, but after boiling and partial aeration it was 8.4. The amplitude of a strip beating in this water decreased at first with a tendency to form waves. Then for a period of 80 minutes the amplitude remained constant, but at the end of this period the amplitude began to increase, becoming 65 per cent greater than that during the previous period of uniform amplitude. Periodicity appeared in about two hours from the beginning of the experiment, the number of beats in each period ranging from ten to sixteen, while the period of interruption varied from one minute and a quarter to three minutes. The pH was still 8.4 about forty-five minutes after periodicity and augmentation were well developed. Apparently these phenomena were not due to increased acidity of the surrounding medium, nor was the cessation of beat in the cases of extreme oxygen lack due to an increase in the concentration of the salts resulting from boiling.

When 75 cc. of boiled sea water were mixed with 25 cc. of unboiled water, the pH of the mixture was 8.8. The first few contractions increased in amplitude about 20 per cent, lasting for about three minutes. Then a fall occurred, and the amplitude remained uniform in height until waves in amplitude appeared in one hour indicating the onset of periodicity, which became well marked about half an hour later. At this time the pH was still 8.8.

Effect of Potassium Cyanide.—When ten drops (about 0.5 cc.) of $M/10$ potassium cyanide were added to 100 cc. of sea water in which a strip had been beating for a few minutes, the results were similar to those obtained with partial aeration. An increase in amplitude occurred within two minutes which varied in different cases from 18 to 400 per cent. The tone increased at about the same time. Periodicity occurred within fifteen minutes. In one case it began in three minutes, and the rate of beat was increased about one beat per minute for a brief period after the addition of potassium cyanide. Finally the tone and amplitude fell and the strip ceased to beat (see Fig. 6).

An examination of the results presented above suggests that lack of oxygen is responsible for the appearance of the two chief phenomena noted. Since augmentation and periodicity did not occur with a continuous flow of water but did occur in three hours when the amount

of water was limited to 100 cc., one might expect that one or more of several factors were responsible, such as, an increase in carbon dioxide, an increase in unoxidized acids, a depletion of essential ions, or a depletion of oxygen. However, when the carbon dioxide content of sea water was increased at the beginning, the phenomena did not

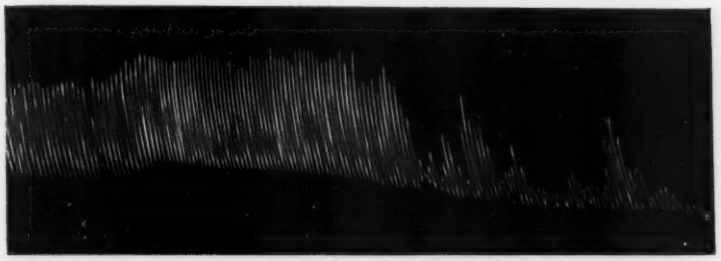

FIG. 6. Experiment 82. Cloacal strip in 100 cc. of sea water. Time of immersion 4:28. At *X*, 4:35, 20 drops of *M*/10 potassium cyanide added.

appear, although a temporary increase in amplitude and tone could be produced upon addition of hydrochloric acid. Moreover, in the experiments in which the phenomena did appear, the pH of the sea water was either unchanged or decreased very slightly. Since both augmentation and periodicity were made to appear much sooner when the water was partly depleted of oxygen at the beginning, or when potassium cyanide was added, the inference is that oxygen lack was either directly or indirectly responsible.

When a ring preparation made from the cloaca of *Cucumaria frondosa* was allowed to beat in a limited volume of sea water, namely, 25 or 30 cc., periodicity appeared in 50 minutes on the average in eight out of ten preparations. Two showed no periods. Augmentation of amplitude occurred in five cases. When boiled sea water was used the periodicity appeared in 25 minutes on the average in 14 out of 15 preparations. One showed no periods. Augmentation of amplitude occurred in 13 preparations. These results, especially when considered in the light of the results on *Stichopus,* indicate that lack of oxygen is a factor tending toward an early development of periodicity and augmentation.

DISCUSSION

Periodicity is a part of the normal respiratory sequence of a holothurian, the rhythmical contractions of the cloaca being inhibited while the body muscles squeeze out the sea water from the respiratory tree

through the relaxed anal valve. Crozier (1916), however, found no evidence of periodicity in the cloacal-end preparation of *Stichopus* and came to the conclusion that the stimulus for spouting has its origin outside the cloaca. It should be noted that he used larger volumes of water than were used in the work reported in this paper. Apparently no oxygen deficiency existed in his preparations, in which the cloacal pumping probably produced a sufficient movement of water to keep it aerated beyond the needs of the preparation. Since the isolated cloacal strip will exhibit regular periods of inhibition, the inference is that a part of the normal mechanism for spouting lies within the cloacal muscle. Since periodicity is lacking with sufficient aeration and appears quickly under conditions of oxygen deficiency, one is inclined to believe that low oxygen tension is a factor in determining the normal respiratory sequence in the holothurian.

Periodicity is commonly observed in the respiratory activity of vertebrates, as for example in the breathing of hibernating animals, in Cheynes-Stokes respiration, and in respiration at high altitudes. The causes of this phenomenon are usually associated with the chemical conditions in the respiratory center. Most authors have offered explanations which concern directly or indirectly the hydrogen ion concentration of the blood or fluid surrounding the cells. Gesell (1925), however, has called attention to the hydrogen ion concentration within the cells of the former, pointing out that when oxygen is present carbon dioxide is formed, but if oxygen is lacking lactic acid results. In either case the activity of the center increases as the acidity rises. Assuming a critical level, one needs further to call upon a mechanism for altering either the level or the acidity to account for periodic inhibition.

The augmentation of amplitude observed with a decrease in the available oxygen is in accord with the work of Magnus (1904) and of Frey (1923), who worked on vertebrate smooth muscle. Gross and Clark (1923), in an investigation on the influence of the oxygen supply on the response of the isolated intestine to drugs, stated that cutting off the oxygen resulted in a decrease in amplitude and tone. They did not comment on the immediate brief increase in amplitude and tone shown in their published graphs. The literature offers many additional observations which indicate that a certain degree of oxygen lack results in increased activity of tissue. Kaya and Starling (1909) found that lowering the oxygen tension resulted in excitation in the whole nervous system. Sherrington (1910) found that a certain degree of asphyxia favored the elicitation of the scratch reflex, and suggested that the hyperexcitability of the reflex was due to oxygen

lack. Mathison (1911) showed by the use of hydrogen, nitrogen, and carbon monoxide that the initial effect of oxygen lack on the medullary centers is clearly stimulating. Gasser and Lovenhart (1914) found by the use of carbon monoxide and sodium cyanide that decreased oxidation stimulated the medullary centers at first but later depressed them. Kellaway (1919) demonstrated that lack of oxygen may lead to stimulation of the adrenal glands, and Lutz and Schneider (1919) have observed a dilatation of the pupil in men during a period of breathing nitrogen. They also presented evidence to show that the cardiac and the respiratory medullary centers in man respond very quickly to changes in the partial pressure of oxygen. A decrease in oxygen tension increased the activity of these centers, while an increase in oxygen tension decreased their activity. Glazer (1929) found that intravenous injection of sodium cyanide in a dog increases the reflex response of the anterior tibial muscle, and Winkler (1929) obtained a similar effect with low alveolar oxygen tension.

In the muscle-and-nerve-net preparation reported in the present paper, it appears that the carbon dioxide content and the acidity of the surrounding fluid are not primary factors in controlling its activity. This conclusion is supported by the work of Hogben (1925) who found that, on adding acid to the perfused heart of *Maia* and of *Homarus,* the pH could be lowered from 7.0 to 5.6 without producing a change in the mechanical phenomena. Reduction beyond this point produced an immediate effect on the character of the rhythm. Nor could any alteration be noticed in the beating of the smooth muscle of *Helix* and of *Aplysia* on changing the pH from 7.0 to 6.0. In fact it is possible that the pH outside of the cell may vary markedly without greatly altering that inside of the cell. The oxygen tension appears to have some influence on tissue acidity. Frey (1923) presented evidence which shows that without oxygen the tissue rather than the surrounding fluid first changes its hydrogen ion concentration, and if this approaches the optimal value, an increased ability to respond ensues. The anaerobic production of acid in cellular activity and the rôle of oxygen in the recovery process suggest that oxygen lack is acting indirectly when cellular activity is first increased and is subsequently depressed.

Summary

1. An isolated muscle strip from the cloaca of *Stichopus moebii* Semper and a ring of muscle from the cloaca of *Cucumaria frondosa* were used in sea water as rhythmically beating preparations.

2. In a continuous flow of sea water the contractions (*Stichopus*) were nearly uniform in rate and amplitude over a period of several hours, but a gradual decrease in both finally occurred.

3. In a limited volume of sea water (100 cc.) the amplitude (*Stichopus*) began to increase after three hours and a distinct periodicity of the regular rhythm developed. In the case of the cloacal ring of *Cucumaria* beating in 25 or 30 cc. of sea water, periodicity appeared in 50 minutes on the average.

4. In boiled sea water the strip (*Stichopus*) ceased beating in from three to five minutes, but partial recovery took place if the water was aerated within three minutes. If the carbon dioxide was replaced in the boiled sea water, cessation of beat occurred as before. In 25 or 30 cc. of boiled sea water the ring of cloacal muscle from *Cucumaria* developed periodicity in 25 minutes on the average.

5. A moderate excess of carbon dioxide in sea water (pH 7.0) did not bring on augmentation nor produce periodicity.

6. In boiled sea water of various degrees of aeration the augmentation of amplitude and the periodicity appeared sooner than in unboiled sea water. When little oxygen was present both phenomena appeared almost immediately, while the pH of the surrounding fluid was unchanged.

7. When potassium cyanide was added to the sea water an increase in amplitude and tone occurred and periodicity appeared.

8. Evidence from the literature is cited supporting the view that decreased oxygen tension results at first in increased activity of muscular and nervous tissues. This view is further supported by the evidence presented in this paper.[2]

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PHENOTYPICAL VARIATION IN BODY AND CELL SIZE OF *DROSOPHILA MELANOGASTER*

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I.

The purpose of this paper is to contribute to the solution of the question of the relationship of the cell size and body size, using well-known and standard material. The literature devoted to this question is very extensive, but most of the work done cannot be considered to fulfil the requirements of exact experimental investigation, in regard either to the control of conditions, or the homogeneity of the material, or the precision and accuracy of the treatment. Comparatively modern compilations of the data available have been made by Levi (1906) and Martini (1924).

Concerning the more limited problem of the correlation of body size and cell size in Diptera there have been two recently published papers. Loewenthal (1923) attacks a problem which corresponds to one part of the present investigation, namely the influence of underfeeding on the body and cell size of the blow-fly. The first criticism which may be made of Loewenthal's work is that he does not give any indication of the ages of the normal and underfed maggots. It therefore is not clear whether the observed smaller size of the hypodermis cells is due to differences in the age of larvae or in the feeding. At the same time Loewenthal does not find any difference in the cell size of the gonad rudiments, in spite of their difference in size. The following conclusion is reached (p. 91): " Danach ist die Körpergrösse der ausgebildeten Imagostadiums unabhängig von der Zellgrösse und allein bedingt von der mehr oder minder grossen Zellanzahl." Further a totally incorrect statement is made concerning the absence of cell divisions during the larval life (p. 92): " Mit Abschluss der Embryonalentwicklung stellen die larvalen Zellen ihre Vermehrungstätigkeit ein, das ganze Wachstum der Larve von wenigen mm Länge nach dem Schlüpfen aus dem Ei bis zur Länge von 2 cm einer verpuppungsreifen Ruhelarve beruht allein —wenn man von den während der Larvalperiode für die Gesamtgrösse nicht ins Gewicht fallenden Imaginalanlagen absieht—auf dem Grössenwachstum der Zellen." Przibram's and Megušar's (1912) investigations showed that this is not the case in the postembryonal develop-

ment of *Sphodromantis* (Orthoptera, Mantidae) and I (1929) have shown also that the metamorphosis of *Drosophila* is connected with six simultaneous divisions of the cells of the whole body.

The same subject of the relationship of the size of an organ and the size of the cell has been touched upon by Bridges (1921, 1925). In both of his papers differences in the cell structure, namely, nuclear structure, are shown to be connected with the size of the whole body and its organs. It was discovered that these intersex-producing females (triploid) could be identified by their somatic characters, namely, large coarse bristles and large roughish eyes (1921, p. 253). In the second paper it is the size of the ommatidia which is shown to be different in flies having different chromosomal complexes. "The cells of triploid individuals are readily seen to be larger than the cells of diploids, and correspondingly their facets are larger" (Bridges, 1925, p. 709).

I became interested in the problem of body size and cell size years ago while working on the oceanographic expedition of the Floating Marine Scientific Institute to the Russian arctic seas. The first expedition in 1921 gave very impressive material on the geographical variation in the dimensions of the body of different marine animals. It could be particularly easily shown on such a group of animals as Isopoda, which have a postembryonal development ending with a definite imaginal stage analogous to that of insects. Extensive biometrical data on variation of Isopoda, taken from localities with different temperatures, showed perfectly that the colder regions (for instance, the Kara Sea) are populated by races which have a larger body size than regions with warmer water temperature (Barents Sea). On the second expedition I strove to collect some material on the histology of local races of some of the species of Isopoda. But the severe conditions of navigation during this and following summers did not allow the accomplishment of this intention. During the winter of 1927–28, working at this Institute, I succeeded in working out a more or less accurate method of producing *Drosophila* imagoes of different sizes, using two factors, temperature and underfeeding. The method of counting the number of hairs on the wings of *Drosophila* as a method of estimating the number of cells on a certain surface of the wing was discovered by a friend, Dr. Th. Dobzhansky (1929), who was kind enough to explain it to me. I have the pleasure to express also my deepest gratitude to Dr. Raymond Pearl for criticism and valuable suggestions.

II.

Two factors have been used in producing flies of an abnormal size. It was shown in an earlier paper that the first of them was the low tem-

perature, which decreases the rate of development and produces flies of a larger size (see Alpatov and Pearl, 1929). The method of collecting new-born larvæ has already been described (Alpatov, 1929). Flies belonging to Wild Line 107 have been taken for parents of our experimental animals, the collected larvæ being 0–2 hours old at the moment of putting them on food. The bottles had been planted with yeast 2 hours before the putting on of larvæ, and watered with a few drops of distilled water. Electric and low temperature Hearson incubators were used for keeping the bottles with flies. Five bottles with 50 larvæ each were kept at 18° C., five others at 28° C. The development from the moment of the populating of the bottle till the moment of the pupation was more than twice as long in the cold series as in the warm. It is unnecessary to discuss here at length the question of temperature and development rate, this having been done in another paper (Alpatov and Pearl, 1929). The technique of breeding in the experiment with underfeeding was the same except for the fact that the yeast was put in the bottles with synthetic medium at the moment of populating the bottles with larvæ.

A method of getting undernourished larvæ by taking larvæ from the food before the normal end of feeding has been used by various workers, for instance, Ezhikov (1917, 1922), Smirnov (1926, 1927), Cousin (1926), Herms (1928) and others. Most of these authors did not attempt to determine with sufficient accuracy the moment of taking the larvæ from the food, Herms being in that respect an exception. In the present investigation, larvæ were taken from the food exactly 48 hours after the moment of populating the bottles with 0–2 hour-old larvæ. Larvæ which reached the desired age were taken from bottles and placed in half-pint bottles containing plain agar. The mouths of the bottles were covered with 40 mm. watch glasses and sealed with plastaline used in modelling. This was done in order to prevent the larvæ, which become very active, from crawling out. The day after the larvæ had turned into pupæ the watch glasses were replaced by the usual cotton stoppers.

Table I shows that the larvæ with a subnormal period of feeding pupate earlier than normally fed ones. This can be compared with Kopeč's (1924) statement that ". . . if we begin to apply starvation to older specimens during developmental stages . . . the transformation of these animals is accelerated." A little longer prepupal development of the normally fed larvæ, those which served as controls to the underfed being compared with the 28° flies of the early October experiment, cannot be very easily interpreted. It might exist in a difference in conditions—perhaps a difference in yeast growth which lengthened the duration of development of larvæ in the second set of experiments.

TABLE I

Data on the Conditions of the Development of Flies Reared for the Study of the Problem of Cell Size

	Temperature limits of variation	Average	Time of the beginning of the experiment	Time from egg until pupation, in hours	Time of feeding
Underfed flies	Kept at 28°	—	October 24, 1928	80.39±.50	48 hours
Normally fed flies	Kept at 28°	—	October 24, 1928	93.16±.74	Until normal leaving of the food
28° flies	27.1–28.9°	28.2°	October 8, 1928	87.40±.36	Until normal leaving of the food
18° flies	17.0–20.0°	18.2°	October 8, 1928	200.86±.89	Until normal leaving of the food

The flies have been collected in 70 per cent alcohol and measured in glycerine under a cover glass. The following characters on the wings of collected flies have been studied: the length and width of the wing, and the number of hairs on a surface equal to 0.1 square mm. on the lower surface of the wing. Fig. 1 represents the points of measurement and the place where the hairs have been counted.

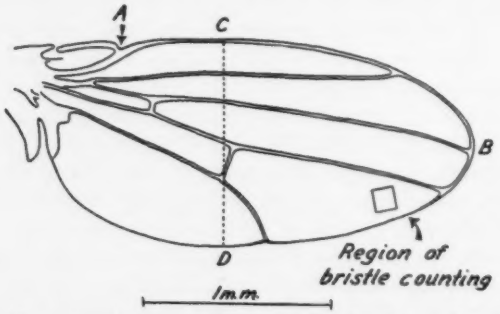

FIG. 1. Measurements of the wing. *AB*, length of the wing, *CD*, width of the wing. The square shows the area of the bristle countings.

For the measurements the following optical systems were chosen: Spencer 25.4 mm. objective and a micrometer ocular in a No. 2 ocular. The countings of the hairs of the lower surface of the wing were done in a way approved by Th. Dobzhansky. Pieces of paper with squares

representing 0.1 square mm. at a given magnification have been prepared by projecting through an Abbé camera lucida 0.1 mm. from an object micrometer placed on the microscope stage. A Spencer microscope was used with objective 4 mm. and ocular × 10. The hairs have been projected by means of the camera lucida and drawn with a sharp pencil. Only hairs whose bases happened to fall inside the square have been counted (Fig. 2).

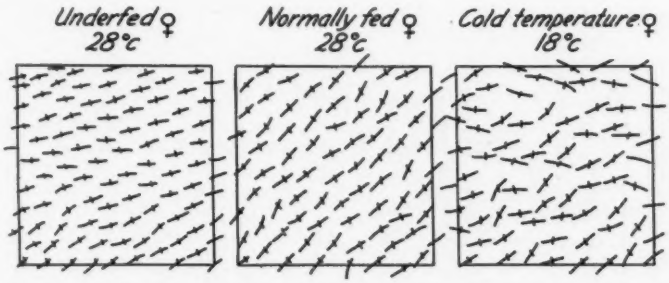

FIG. 2. This figure represents the bristles on the surface of 0.1 mm.² in the lower surface of the wings of underfed, normally fed, and cold temperature females. The bristles which have a line across their middle have been counted, those without lines had their basis outside the limits of the 0.1 mm.² and have not been counted.

We did not consider it wise to count the hairs exactly at a certain point (in so many parts of a millimeter from a certain vein) as has been done by Dobzhansky. There are two reasons for not doing so. First of all the distribution of hair on that part of the wing is more or less uniform. On the other hand, the wings of underfed and normal are so different in size that a distance expressed in absolute measurement would show morphologically quite different regions. Fifty specimens of each set of underfed, normal fed and 18° flies were studied in regard to the density of the hairs. Dr. Th. Dobzhansky succeeded in finding that on the wings each hair corresponds to a separate cell. This can be seen on specimens of flies just emerged from the pupæ. The wings look opaque and the cells can be distinctly seen. It is very likely that the tiny hair covering the thorax of *Drosophila* corresponds also to hypodermis cells, and their density may also be used as a method of studying the size of the hypodermal cells.

III.

It is desirable at this stage to digress briefly to consider a matter which arose as an extension of the original problem. It is the question

of functional relation between the time of larval feeding and the final size of the flies. First of all I reinvestigated the data published by Herms (1928) and found that when plotted on a diagram they reveal a very interesting picture.

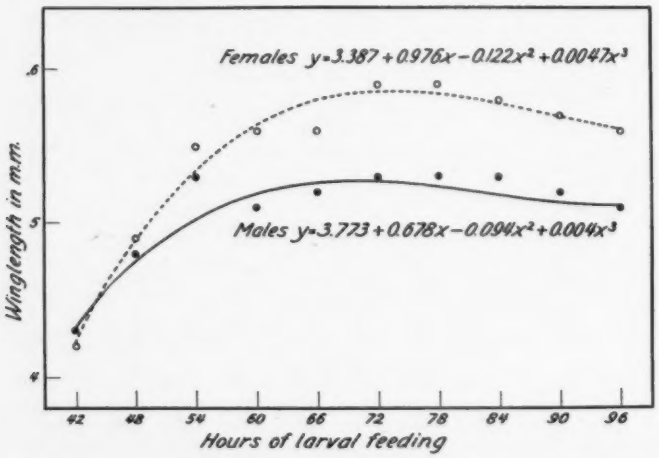

FIG. 3. The relation between the wing length and the length of the feeding period in *Lucilia sericata*. Data from Herms (1928).

Fig. 3 represents Herms' data and two cubic parabolas which I fitted to the observed points. Up to the 78 hour point the trend of the curves represents the upper part of a typical growth curve. There cannot be any doubt that this trend corresponds exactly to the upper branch of the logistic curve which can be fitted to the growth of *Drosophila* larvæ of the third instar (see Alpatov, 1929). But the decline after 78 hours is quite remarkable. Going back to my paper on larval growth in *Drosophila* I was able to find in Fig. 13 particularly a slight indication as to an analogous decline of the size of the larvæ killed at the latter end of the life of the culture. It was therefore decided to clear up this question on specially collected material. This was done in April 1929. Forty bottles containing 0.500 grams of Magic yeast with 25 drops distilled water were populated by 80 larvæ each. Five drops of water were added every day during the larval growth. The experiment was run at a temperature of 25° C.

Table II contains data on the sex relations in the material studied. Let us first compare the percentage of males emerged from larvæ taken from the food at 48–80 hours, which is equal to 102.6, with that of

males emerged from larvæ taken from food at the age of 84–96 hours, in which case the percentage is 89.9. This difference finds its explanation in the fact that male larvæ in our case started pupation earlier than females, which is shown by the very high percentage of males among

TABLE II

Absolute and Relative Numbers of Larvæ, Pupæ and Adult Flies in the Experiment on Underfeeding of Larvæ

Hours from the beginning of feeding	Number of larvæ taken from the food	Number of the pupæ observed	Number of pupæ unable to produce flies	Number of flies emerged				
				Total	In per cent of the larvæ	Male	Female	Male in per cent of female
48	151	—	17	30	19	18	12	150.0
52	170	—	7	80	47.1	44	36	122.2
56	152	—	11	87	57.2	37	50	74.0
60	158	—	12	123	77.8	62	61	101.6
64	162	—	2	154	95.1	72	82	87.8
68	170	—	7	153	90.0	85	68	125.0
72	151	—	12	117	77.6	69	48	143.8
76	135	—	3	127	94.1	56	71	78.9
80	150	—	1	142	94.7	70	72	97.2
Total 48–80	—	—	—	—	—	513	500	102.6
84	86	36	2	78	90.7	39	39	100.0
88	137	12	4	115	83.9	62	53	117.0
90	111	30	11	100	90.1	56	44	127.3
92	110	46	3	100	90.1	45	55	81.8
94	95	61	10	79	83.2	30	49	61.2
96	85	73	6	75	88.2	27	48	56.3
Total 84–96	—	—	—	—	—	259	288	89.9
Flies emerged from pupæ at 84–96 hours								
84	—	—	—	—	—	25	11	227.3
88	—	—	—	—	—	6	5	120.0
90	—	—	—	—	—	23	8	287.5
92	—	—	—	—	—	30	12	250.0
94	—	—	—	—	—	32	23	139.1
96	—	—	—	—	—	45	27	166.1
Total 84–96	—	—	—	—	—	161	86	187.2

larvæ pupated naturally at the age of 84–96 hours—187.2 per cent. On the whole the group of bottles which was taken to get larvæ fed 84–96 hours shows a percentage of males equal to 109.1. Comparing it with the sex proportion in normal undisturbed bottles where we had

356 males and 415 females, we find that the normal percentage of males is 85.8. We can therefore draw the conclusion that there is a definite preponderance of males among flies emerged from the underfed larvæ. In other words it seems that a selective process makes the male more resistant to underfeeding.

TABLE III

Wing Length, Width and Relative Width of the Flies Emerged from Larvæ taken from the Food at Different Hours

Hours	Males				Females			
	Length	Width	Index	Number	Length	Width	Index	Number
48	1.107	.6490	58.6	17	1.207	.6972	57.8	12
52	1.164	.6847	58.8	25	1.239	.7154	57.7	25
56	1.331	.7847	59.0	25	1.413	.8034	56.9	25
60	1.321	.7697	58.3	25	1.493	.8459	56.7	25
64	1.394	.8145	58.4	25	1.572	.8898	56.6	25
68	1.409	.8289	58.8	25	1.588	.9102	57.3	25
72	1.406	.8428	59.9	25	1.561	.9083	58.2	25
76	1.371	.7983	58.2	25	1.511	.8493	56.2	25
80	1.412	.8261	58.5	25	1.586	.8938	56.4	25
84	1.476	.8833	59.8	25	1.673	.9349	55.9	25
88	1.440	.8516	59.1	25	1.641	.9321	56.8	25
90	1.472	.8777	59.6	25	1.646	.9255	56.2	25
92	1.423	.8468	59.5	25	1.614	.9032	56.0	25
94	1.426	.8457	59.3	25	1.613	.9077	56.3	25
96	1.444	.8686	60.2	25	1.608	.9083	56.4	25

TABLE IV

Wing Length, Width and Relative Width of the Flies Emerged from Pupæ Pupated at a Given Hour, and of Those Emerged from Pupæ Pupated during the Whole Pupation Period

Hour	Males				Females			
	Length	Width	Index	Number	Length	Width	Index	Number
84	1.484	.8805	59.3	24	1.709	.9901	57.9	11
88	1.490	.8887	59.6	6	1.728	.9944	57.5	4
90	1.493	.8876	59.5	15	1.715	1.007	56.7	8
92	1.456	.8516	58.5	25	1.649	.9312	56.5	12
94	1.455	.8499	58.4	25	1.644	.9389	57.1	23
96	1.463	.8544	58.4	25	1.672	.9536	57.0	25
Normal pupation	1.475	.8745	59.3	40	1.673	.9668	57.8	40

Tables III and IV give the average length and width of wings of our material. The wing length is graphically represented in Fig. 4.

With the exception of some cases (72, 76 and 80 hours) the material confirms what could be seen on curves based on Herms' data. The most interesting thing is the declining slope of the curves toward the end. It is not only with underfed flies that this decline is noticeable,

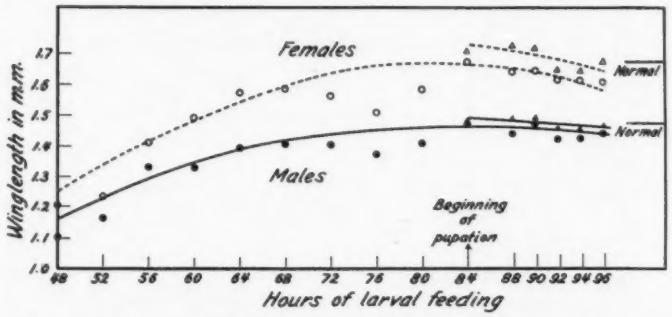

FIG. 4. This figure represents the relation of the length of the wing and the length of the larval feeding in *Drosophila melanogaster*. The triangles indicate the length of wings of flies pupated at certain hours.

but the flies normally pupated in the beginning of the pupation period had longer wings (*i.e.*, larger bodies) than flies in which pupation has been delayed.

Table V gives the statistical proof of this conclusion. It can be definitely seen that in males and females without regard to whether the pupation is going naturally or the flies emerge from larvæ taken from the food, those which pupate first are larger than those which pupate later. We may express the observed phenomenon in a little different form. There is a negative correlation between the duration of larval life and the size reached during growth. The faster the larva grows the sooner it reaches the pupal stage. We take the liberty of comparing our case with the experiments on *Cucumis melo* described by Pearl in his book, *The Rate of Living*, (1928). The larvæ which reach a larger size in a short time have naturally a higher rate of growth than larvæ which remain small for a longer time. Therefore the statement brought forward by Pearl (p. 139) that "between growth rate and duration of life to the beginning of death the correlation is negative and significant in degree" can be perfectly well applied to our case.

We do not know whether these differences arise really as a result of inherent vitality or are the result of differences of treatment of larvæ during the population of the bottle. Further experiments have to solve this question. Our results are very close to Kopeč's discovery (1924) of the negative correlation between the duration of larval period

TABLE V

Length of the Wings (in mm.) of Flies Emerged from Larvæ Taken at a Given Hour from the Food, from Pupæ Formed at a Certain Hour, and from Control Bottles

Males

			Difference and ratio			
Larvæ taken from the food at 84–88 and 90 hours. $N = 75$	Mean	1.458±.004	0.27±.005	Larvæ taken from the food at 92–94–96 hours. $N = 74$	Mean	1.431±.003
	Standard deviation	.0450	R=5.4		Standard deviation	.0405
	C. of V.	3.08±.17			C. of V.	2.83±.16
Difference and ratio	—	.025±.005 R=5.0	—	Difference and ratio	—	.026±.004 R=6.5
Pupated at 84–88–90 hours. $N = 45$	Mean	1.433±.003	.022±.004	Pupated at 92–94–96 hours. $N = 74$	Mean	1.457±.002
	Standard deviation	.0265	R = 5.5		Standard deviation	.0277
	C. of V.	1.79±.13			C. of V.	1.90±.11
—	—	—	—	Pupated during the whole pupation period. $N = 40$	Mean	1.469±.004
					Standard deviation.	.0393
					C. of V.	2.68±.20

TABLE V—*Continued*

Females

			Difference and ratio			
Larvæ taken from the food at 84–88–90 hours. N = —	Mean	1.650±.004	.043±.005	Larvæ taken from the food at 92–94–96 hours. N = 75	Mean	1.607±.003
	Standard deviation	.0545			Standard deviation	.0432
	C. of V.	3.30±.18			C. of V.	2.69±.15
Difference and ratio	—	.064±.006 R=10.7	—	Difference and ratio	—	.047±.004 R=11.75
Pupated at 84–88–90 hours. N = —	Mean	1.714±.004	.060±.005	Pupated at 92–94–96 hours. N = —	Mean	1.654±.003
	Standard deviation	.0312			Standard deviation	.0339
	C. of V.	1.82±.18			C. of V.	2.05±.13
—	—	—	—	Pupated during the whole pupation period. N = —	Mean	1.672±.005
					Standard deviation	.0507
					C. of V.	3.03±.23

and the weight of the chrysalids in *Lymantria dispar* (L.). This negative correlation found in twelve experimental groups out of sixteen is particularly well expressed in males.

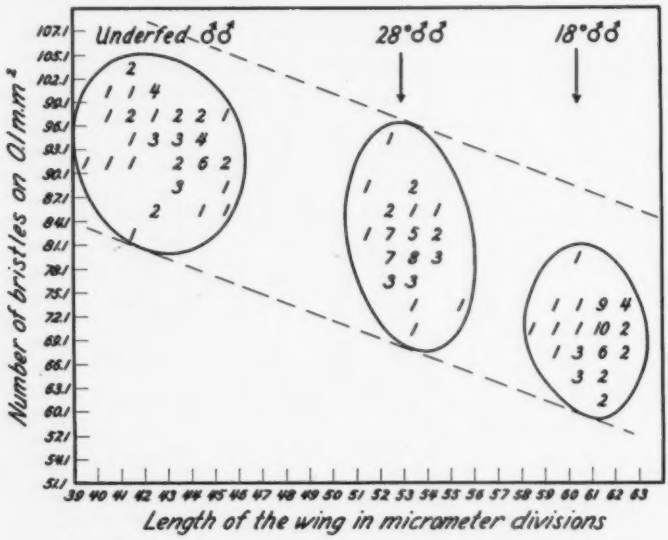

FIG. 5. Correlation between the length of the wings and the number of bristles per 0.1 mm.² on the lower surface of the wings of the male.

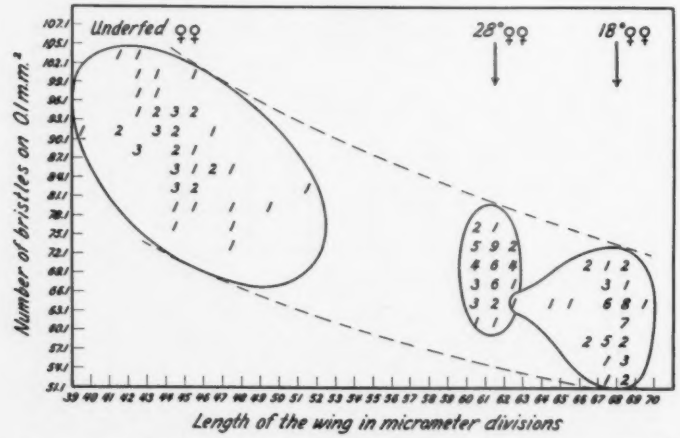

FIG. 6. Correlation between the length of the wings and the number of the bristles per 0.1 mm.² on the lower surface of the wings of the females.

TABLE VI

Biometrical Constants for the Length of the Wings and the Number of the Bristles per 0.1 mm.² of the Lower Wing Surface, as Well as the Coefficient of Correlation between These Characteristics

Males

	Underfed flies		Normally fed flies		28° flies		18° flies	
	Number of the bristles per 0.1 mm.²	Length of the wing in mm.	Number of the bristles per 0.1 mm.²	Length of the wing in mm.	Number of the bristles per 0.1 mm.²	Length of the wing in mm.	Number of the bristles per 0.1 mm.²	Length of the wing in mm.
Mean	94.00±.46	1.224±.004	81.04±.39	1.507±.002	—	1.488±.002	70.00±.34	1.737±.002
Standard deviation	4.836	.0456	4.125	.0230	—	.0227	3.600	.0240
C. of V.	5.14±.35	3.73±.25	5.09±.34	1.53±.10	—	1.53±.10	5.14±.35	1.38±.09
r	−0.299±.086 *R* = −3.5		−0.024±.094				+0.098±.093	

Females

Mean	89.14±.68	1.283±.006	70.36±.35	1.737±.002	—	1.716±.002	63.50±.46	1.917±.005
Standard deviation	7.089	.0590	3.691	.0190	—	.0215	4.773	.0498
C. of V.	7.95±.54	4.59±.31	5.25±.35	1.09±.07	—	1.25±.08	7.52±.51	2.60±.18
r	−0.563±.064 *R* = −8.8		+0.075±.094				+0.101±.093	

IV.

Correlation tables shown in Figs. 5 and 6 contain the basic data on the number of hairs on 0.1 mm.^{2} and length of the wings. The horizontal axis gives the wing length in divisions of the ocular micrometer, each division being equal to 28.333 microns. Table VI represents constants derived from Figs. 5 and 6 with the addition of wing length of 28° flies. The wing length is expressed in millimeters.

TABLE VII

Average Width of the Wings and Width Index, i.e., Width Expressed in Per Cent of the Length

	Underfed flies		Normally fed flies		28° flies		18° flies	
	Width of the wing	Index	Width of the wing	Index	Width of the wing	Index	Width of the wing	Index
Males . . .	.7151	58.23	.8970	59.33	.8871±.0021	59.62±.11	1.024	58.85
Females .	.7253	57.29	1.015	58.30	1.004 ±.002	58.51±.12	1.102	57.31

Let us discuss the influence of the factor under consideration on the wing as a whole. Table VII gives us the constants for the width in millimeters as well as the width in percentage of the length. There is a pronounced sex difference in the size of the wing, the females in all groups being larger than the males. The relative width of the wing is larger in the males, as can be seen by comparing males and females in all groups, and particularly those of the 28° group. The difference is 6.9 times larger than its probable error. (The indices in this case have been calculated by the use of Pearson's formula.) Another point of interest concerning the relative width of the wing is that in the females as well as in the males the underfed and 18° flies seem to have narrower wings than the "normal" 28° flies. The sex difference is also influenced by abnormal conditions. Table VIII shows that in "normal" 28° conditions the sex difference is the greatest, while underfeeding and low temperature reduce the difference. The lower line in Table VIII contains recalculated data from the experiment described in a former paper (Alpatov and Pearl, 1929). The effect of low temperature and consequently of the slow development can be seen in this case also. It is difficult to find an adequate explanation of this phenomenon, which very likely is connected with certain differences in male and female postembryonal development, that is, with different time of the manifestations of different characters during the larval or pupal life.

Turning our attention to the main problem of our investigation, one glance at the correlation tables shows that the larger the size of the wing of the corresponding group of flies, the smaller the number of cells on the area of 0.1 mm.^{2} In other words, the larger flies, considering *inter-group* variation, have also larger cells. The coefficients of correlation for each of the six groups of flies have been calculated separately. They are given in Table VI. Only in the case of underfed males and females is the correlation significant and negative. The conclusion is that in underfed flies the size of the body is negatively correlated with the number of cells on a definite surface of the wing. A possible but very dubious explanation of the absence of such correlation in the case of normally fed and cold temperature flies might be that the variation in the wing length of *Drosophila* developed from normally fed larvæ is so small that the correlation could not manifest itself.

TABLE VIII

Sex-Index of the Wing Length, i.e., Male Wing Length Expressed in Per Cent of the Female

When studied	Underfed flies	Normally fed flies (28°)	28° flies	18° flies
1928	95.33±.54	86.76±.15	86.71±.15	90.61±.26
1927	—	—	88.18±.16	91.93±.18

So far as the variation of the flies belonging to different groups is concerned, it can be seen that the coefficient of variation of the number of cells does not show any definite difference in different groups. At the same time the variation of underfed flies in the length of the wing is much greater than that of the flies which had a normal feeding, no matter at what temperature. Previous investigators who have worked on variation of flies under conditions of under-feeding have also described the increasing variation of experimental animals (see Smirnov and Zhelochovtsev, 1926).

We have now to approach the problem of the actual surface-size of the cells and its relationship to the size of the whole organ. Table IX represents all the calculations relating to this question. The surface of a cell in square microns was determined by dividing 10,000 microns (0.1 mm.^{2}) by the number of hairs on that surface. It can easily be seen that the larger the flies the greater the surface of the cell. Another point of interest is the pronounced sex difference in the size of the cells, the females having much larger cells than the males. This has been pointed out by Dobzhansky (1929). The next step was to

TABLE IX

Biometrical Constants and Indices for Different Characteristics of the Experimental and Control Flies. The last three columns show these characteristics expressed in percentages of the values for the 18° flies.

Character	Underfed flies	28° flies	18° flies	Underfed flies	28° flies	18° flies
Males						
Surface of a cell	106.32±.52	123.40±.59	142.86±.69			
Square root of the surface = "length" of a cell	10.31±.07	11.09±.08	11.95±.08	86.3	92.8	100
Calculated length of a cell	8.42±.08	10.37±.08	11.95±.08	70.5	86.8	100
Length of the wing	1.224±.004	1.507±.002	1.737±.002	70.5	86.8	100
Females						
Surface of a cell	112.18±.86	142.13±.71	157.48±1.14			
Square root of the surface = "length" of a cell	10.59±.09	11.92±.08	12.55±.11	88.8	95.0	100
Calculated length of a cell	8.40±.10	11.37±.08	12.55±.11	66.9	90.6	100
Length of the wing	1.283±.006	1.737±.002	1.917±.005	66.9	90.6	100

come from the surface values to linear values which has been done by calculating the length of the cell, which was obtained by taking a square root of the surface of the cell. The data on wing length gave the possibility to calculate the percental decrease in the wing length taking the wing length of 18° flies as a basis. Multiplying by this percental decrease in wing length the number giving the "length" of cell in cold temperature (18°) flies, we obtained the figures represented in our table under the heading "Calculated length of cells." Comparing them with the dimensions obtained by taking the square root, we can easily see that the assumption that the wing length varies proportionally to the length of its constituents does not hold true. The three columns on the right of Table VII represent the changes in wing size and cell size expressed in per cent of 18° (cold) flies. The same relationship between these two characteristics is shown in a percental scale on Fig. 7, the diagonal line represents the relationship in case of a proportional change in wing length and cell length; the dotted line shows the actual percental decrease in cell size in different groups of our flies.

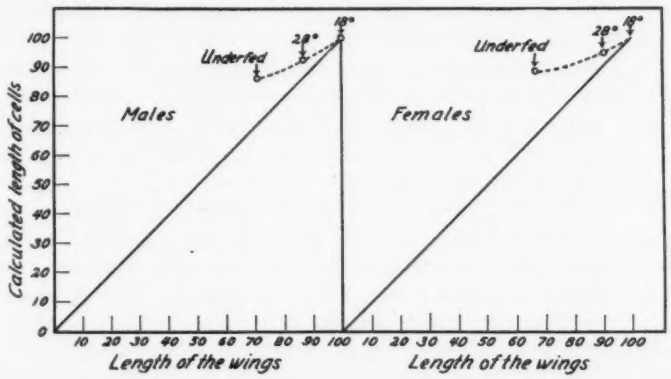

FIG. 7. The dotted lines represent the relationship between the percentage of decrease of the wing length and the percental decrease in the corresponding percental length of the cells calculated by taking the square root of the surface of the cells.

The general conclusion of all these calculations is that the reduced size of cells alone cannot explain the reduction of the organ. The only possible way to explain it is the assumption that the decrease in the organ size—in our case in wing size—is not the result of a decreased size of its cells alone, but also of a reduced number of cells. This last conclusion has a certain bearing upon the problem of the cell constancy in the organism.

If our discussion is correct, the organism can evidently react to the factor decreasing in size not only by decreasing the size of the cells but also the number of cell divisions. The present limited material does not warrant further discussion, but it may be hoped that other investigations in the field of cell-biometry may create a similar basis for understanding the variation of the whole organism as *Die Zelluläre Pathologie* of Virchow did for the interpretation of the pathology of the whole organism.

Summary

1. Dobzhansky's method to determine the number of cells under the surface of the wing membrane of *Drosophila melanogaster* by counting the number of hairs has been used in the present investigation of the relationship of the organ size to the size of its cells.

2. Underfeeding and development at low temperature have been the factors to produce flies under and above the normal size.

3. The functional relation between the time of feeding of larvæ and the size of the wings of larvæ being the expression of the upper part of the logistic larval growth of the third larval instar can be expressed by a cubic parabola.

4. There is a definite tendency for large larvæ (*i.e.*, fast-growing ones) to pupate earlier, which finds a certain analogy with Pearl's correlation that "between growth rate and duration of life (in this case, duration of larval life) to the beginning of death the correlation is negative and significant in degree."

5. As far as all three groups of flies (underfed, normal and cold flies) are concerned the size of the wings is negatively correlated with the number of hairs on a definite surface of the wing when the groups are considered *as wholes* (inter-group correlation). The existence of such a negative correlation could be shown also *within* the group of underfed females and males, but not within the other groups.

6. Expressing in per cent the increase in size of the whole organ and the increase of the linear dimensions of the cells there is a discrepancy in the rate of changes. This leads to the conclusion that the changes in size of the wing cannot be accounted solely by the changes in the size of the cells. The number of cells must play also a certain rôle in this process.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

I. Introduction. A Simple Method for Studying the Rate of Hemolysis

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I.

There is almost no other single type of animal cell which has been so extensively employed in experimental work in the fields of osmotic phenomena and of cell permeability as the mammalian erythrocyte. From the time of the early studies of Hamburger (1886) down to the present day it has been recognized as possessing a number of peculiar advantages as experimental material. Thus, it can be obtained at all times and places in what for practical purposes are unlimited quantities; indeed, the investigator himself carries about with him wherever he goes a never-failing supply of absolutely fresh and normal erythrocytes, ready for use at a moment's notice. Because of the remarkable constancy of its natural environment—the mammalian body—the erythrocyte, unlike certain other cells frequently used for similar studies, may be expected to show only relatively slight variations in its physiological properties from day to day and from season to season. Furthermore, its simple structure and low rate of metabolism prevent complications which are frequently troublesome with other types of material. Removed from the body it can be kept, if not in an unaltered, at least in a usable condition for a longer time than almost any other kind of animal cell. Finally, there are available for its study methods of great simplicity which are not only quantitative but which are also statistical to an extent perhaps nowhere else realized with physiological material.

Because of these striking, and to a considerable extent unique, advantages the erythrocyte would appear to be an almost ideal type of material for studies in which a high degree of quantitative accuracy is desired. A survey of the literature, however, reveals all too frequently a disappointing failure on the part of investigators to obtain results of this character. Not only is there a very common lack of agreement between the conclusions reached by different workers, but even the same investigator is not infrequently forced to acknowledge an inability on repeating his experiments to obtain consistent and reproducible data.

The erythrocyte, in spite of its apparent simplicity, behaves, in fact, as if it were either naturally a highly variable and capricious type of material, or—what is more likely—as if it were peculiarly sensitive to certain environmental factors which with other types of cells are much less troublesome.

In the course of work which has occupied the author for several years and which will be reported in detail in the series of papers of which the present one is the first, the general conclusion has been reached that the erythrocyte is indeed a highly suitable form of material for many types of experimental work and that accurately reproducible results may be obtained with it, but that such results are possible only with a more careful attention to details than is needed with most other forms of physiological material. As a matter of fact, the very simplicity of the mammalian erythrocyte, which in its mature condition is perhaps only questionably to be called a living cell at all, prevents the maintenance by it in a changing environment of the relative internal constancy which is so characteristic of more complicated cells and of entire organisms. The simplicity of the erythocyte is, therefore, rather paradoxically, actually a source of complexity for the experimenter. Furthermore, there are certain special reasons, closely connected with the functions which the erythrocyte has to perform, why its osmotic properties, in particular, are of necessity far more profoundly affected by slight environmental changes than are those of perhaps any other known type of cell. These reasons will be discussed in the second paper of this series.

In general, the relation which the erythrocyte, considered as experimental material, appears to bear to other types of cells is much the same as that which a canoe bears to boats of more stable design. Both the erythrocyte and the canoe when properly handled have very definite and characteristic advantages, but both have the tendency to penalize any carelessness in their management in a prompt and unmistakable manner. Perhaps at some future day this peculiarity of the erythrocyte may be considered rather as an advantage than a disadvantage.

II.

Before considering certain of the peculiarities of the erythrocyte itself it seems advisable to deal with some of the methods which have been employed in the past in studying the osmotic properties of this type of cell, and, in particular, with the one which has been gradually developed by the author and has been used in the experimental work upon which all of the papers of the present series are based. By giving

a single description of the method at this point, unnecessary repetitions may later be avoided.

Osmotic changes in the erythrocyte are, in general, always associated with volume changes. This is true whether the changes are of the simple sort produced by the passage of water alone between the cell and its surroundings or of the more interesting and complicated type, so useful in studies of cell permeability, where the movement of water depends upon osmotic inequalities set up by the passage of dissolved substances across the cell boundary. Any quantitative study of osmotic phenomena will therefore involve the measurement of the amount of volume change which occurs in a given experiment, or the rate of this change, or both.

In the case of the erythrocyte there are available two remarkably simple methods for studying volume changes. The first is the hematokrit method introduced by Hedin (1891). By means of it the total volume of all of the cells in a sample of a given suspension is measured, the cells being tightly packed together in a fine graduated tube by centrifugal force. The advantages of this method are, first, its simplicity and, second, its statistical nature, by which the variability of the millions of individual cells is averaged out. Its greatest disadvantage—and this, unfortunately, is a fatal one in many cases—is that the time required to pack the cells into a mass free from intercellular fluid is so great, even with the most powerful centrifugalization available, that the method can be used only to obtain final end points or, at most, to follow volume changes of extreme slowness. For this reason, in the present series of studies, it has been possible to use it only rarely.

A second method, of even greater simplicity, is that of hemolysis. This method, first systematically employed by Hamburger (1886), depends on the fact that when an erythrocyte in swelling reaches a certain volume, which varies not only with the species of animal but also probably with the individual erythrocyte, it loses a sufficient part of its hemoglobin to become invisible, or almost so, both as viewed singly under the microscope or in the aggregate in a suspension in a test tube. In some cases it is possible by appropriate treatment to restore the invisible corpuscles to visibility; in other cases it is not.

The term hemolysis is sometimes applied to the mere disappearance of erythrocytes; at other times it is used to describe their more complete destruction. This double use of the term, while unfortunate, is perhaps unavoidable at present and every author should therefore designate the sense in which he employs it. It will here be used, for convenience, to apply to what for practical purposes is the easier and more certain

end point to observe, namely, the disappearance of the erythrocyte from visibility rather than its more or less complete destruction, concerning which there is usually much greater uncertainty. This usage is further justified by the fact that in "osmotic hemolysis" complete destruction is apparently very difficult to obtain. Thus, Adair, Barcroft, and Bock (1921) were unable with water alone to separate the hemoglobin from the cells containing it sufficiently to obliterate certain effects believed to be due to the cells themselves, though this could be done after the addition of ether, which presumably completed the destruction of the cells.

The hemolysis method for studying the swelling of erythrocytes and, indirectly, therefore, the penetration of dissolved substances, possesses the advantage of extreme simplicity. With no apparatus other than a test tube, very fair ideas as to many problems of cell-permeability may be obtained. The apparatus here to be described refines the method to an extent which permits the experimenter to secure results of a really high degree of accuracy. An even greater advantage of the hemolysis method, however, is that it is available for the study of *rates* of swelling, even in experiments of very short duration. In the present series of papers no experiments of a total duration of less than one second will be reported, but the author has pointed out elsewhere (1927) that a principle used with conspicuous success for another purpose by Hartridge and Roughton (1923) can be adapted to the study of hemolytic processes whose duration is only a fraction of a second as is the case, for example, with the hemolysis of the erythrocytes of the sheep in distilled water. In its adaptability to problems involving rapid rates of swelling, and consequently some of the most interesting problems of cell physiology, the hemolysis method is, in fact, of unique importance.

On the other hand, the method possesses at least two disadvantages which must be frankly admitted and then dealt with as adequately as circumstances permit. The first is that hemolysis may be caused or influenced by various factors other than osmotic ones. The disappearance of an erythrocyte does not necessarily indicate that it has by swelling reached some definite hemolytic volume, V_h, though this is frequently the case. It is important, therefore, that certain control experiments shall always be performed before inferences concerning the rate of swelling are drawn from observations on the rate of hemolysis.

These control experiments may take various forms. Thus, in cases where osmotic factors alone are involved, it should be possible to show: (1) that the substance or substances present in the solution in which hemolysis occurs have no observable hemolytic effect when added in varying amounts, up to and preferably exceeding those employed in the

experiments, to an isotonic solution of NaCl or some similar non-penetrating substance; (2) that the process of hemolysis by a pure solution of the substance in question may be stopped at will at any desired point by the addition in osmotically suitable amounts of NaCl, saccharose, etc.; or (3) that if a solution of NaCl be chosen which is sufficiently hypotonic to cause the hemolysis of some but not all of the erythrocytes in a given sample of blood, the addition of the substance to the partially hemolyzed suspension causes no increase in the degree of hemolysis. The last mentioned test is a very delicate one, though it is somewhat difficult to employ for reasons to be discussed in the following paper of this series.

A second disadvantage of the method is that even in cases where it is reasonably certain that the occurrence of hemolysis is due to the attainment of a definite volume, V_h, this volume represents merely one point on the swelling curve. As compared with the egg of Arbacia (Lillie, R. S., 1916), (McCutcheon, M., and Lucké, B., 1926) whose volume changes can be measured continuously, the erythrocyte appears capable at best of supplying to the investigator only very meagre information about the course of the swelling process.

This disadvantage, however, is not so serious as it might at first sight appear to be. There is reason to believe that the course of the swelling of the erythrocyte can be represented by a fairly simple equation (Jacobs, M. H., 1928) which permits the entire curve to be calculated approximately when one point on it is known. This question will be dealt with more fully in a later paper. Furthermore, in perhaps most experiments, what is desired is not so much the entire curve of swelling as some general measure of the velocity of the swelling process under various experimental conditions, and this may frequently be obtained by a comparison of the times required under the conditions in question to reach the *same* state of swelling in each case. For work of this type the critical hemolytic volume, V_h, when such a volume exists, is a very satisfactory and convenient criterion for comparison.

One important additional point connected with the use of the hemolysis method remains to be mentioned. Both this and the hematokrit methods are statistical in the sense that millions of cells are employed with each. But whereas the latter measures the total volume of all of the cells together without separating them into groups, the former is complicated by the fact that different individual cells hemolyze with different degrees of readiness, and in determining the time of hemolysis, the cells must, in effect, be divided into groups for separate time-measurements. The size of these groups will depend upon the delicacy of the method employed. When a distinction can be made between, for

example, 75 per cent and 76 per cent apparent hemolysis, as is the case with the method about to be described, then the time of hemolysis for the group of cells lying between these limits and consisting of one per cent of the total number may be taken as approximately the arithmetical mean of the times at which the above-mentioned degrees of hemolysis are attained. With a cruder method, or in the region of five or ten per cent hemolysis, where measurements are much more difficult to make, the groups dealt with are of necessity larger and a mere averaging of two times gives correspondingly less accurate results.

Because of the heterogeneous nature of any collection of erythrocytes, it is impossible to speak simply of the "time of hemolysis" for a given sample of blood. Different times must be measured for different groups of cells, or, if desired, a single group may be arbitrarily selected for a given experiment by determining in advance for what particular degree of hemolysis the time shall be measured. In any case, the problem is a much more complicated one than if the blood contained only erythrocytes of uniform physiological properties.

On the other hand, a certain degree of heterogeneity may in some respects be an advantage. Assuming that the different degrees of osmotic resistance of the various cells are dependent chiefly on different individual values of the critical hemolytic volume, V_h, which is a plausible, though as yet an entirely unproved assumption, a possible means is suggested for obtaining more information about the course of the entire swelling curve than could be furnished by a perfectly homogeneous group of cells. The details of such a method still remain to be worked out.

A much more definite advantage of the heterogeneity of a given population of erythrocytes is the following. It is frequently necessary to find a solution of "critical concentration" for a group of cells, *i.e.*, which is just at the point of being able to hemolyze these cells without actually doing so. Cells in such a solution are extremely sensitive test objects for studying the effects of such factors as pH, temperature, etc., as will be pointed out in greater detail in a later paper. If the cells in such a group possessed identical properties, it would require many trials to find the appropriate concentration to the desired degree of accuracy (i.e., to less than 0.001M). With as heterogeneous a group, however, as the erythrocytes in ordinary blood, any concentration within fairly wide limits may be selected with the certainty that there will be present in the blood a group of cells which will exactly "fit" the concentration so chosen. In later papers frequent applications of this principle will be mentioned.

III.

A method suitable for the study by the hemolysis method of the osmotic properties of the erythrocyte should possess the following characteristics. It should allow the degree of hemolysis to be estimated more accurately and more rapidly than the usual laborious and not very exact methods of making cell counts or of making hemoglobin determinations after a preliminary centrifugalization. It should permit the time required for the attainment of a given percentage of hemolysis to be measured accurately, even when the total duration of the experiment is only a few seconds. The usual methods are entirely useless in such cases, and this is perhaps the reason why little work has as yet been done on the rates of any except very slow types of hemolysis. The method should, in the third place, provide not merely for the measurement of the time required to reach some single percentage of hemolysis but for that required for the attainment of many different percentages; otherwise, the heterogeneous nature of a population of erythrocytes may give rise to a type of difficulty that will be discussed in a later paper. Finally, though less essential than the characteristics already mentioned, simplicity of the apparatus itself and convenience in its use would be highly desirable features.

The method here described possesses all of these characteristics. It permits successive determinations of the relative concentrations of cells in different suspensions, as well as of apparent percentages of hemolysis, to be made in a few seconds each, which under favorable conditions are reproducible to one or two per cent. It may be used for the study of all rates of hemolysis where the time measured is more than one second. Furthermore, it permits the measurement not merely of the time required to reach some arbitrarily selected degree of hemolysis but also of the times corresponding to all percentages from zero to upwards of 90 per cent. These measurements, which are extremely easy to make, take the form of permanent kymograph tracings where mistakes in instrumental readings or in the recording of them by the observer are impossible, and where all of the details of the experiment are presented in a way that facilitates ready interpretation. Finally, the apparatus is very simple and inexpensive. A crude but satisfactory form of it can be constructed in an hour out of materials available in any laboratory, and its operation can be mastered in a few minutes. The variety of uses to which it can be put and the degree of accuracy which can be secured with it will be made more evident in the later papers of this series.

In principle, the method is not new. It involves merely the measurement of the turbidity of a suspension of erythrocytes by determining

the maximum depth of the suspension through which the image of the glowing filament of a carbon lamp is visible. It is to be noted that what is observed is a distinct image rather than the total amount of transmitted light, as is the case, for example, with the methods of Ponder (1923, 1927) or with the nephelometer. Methods similar to the present one for the study of suspensions have been used or suggested by Vlès (1921), Holker (1921) and others, but they lack certain of its most useful features.

The source of the image is the filament of an old-fashioned carbon lamp. The brightness of the filament is kept constant by the use of a milliammeter to measure and a sliding rheostat to regulate the current flowing through it. For the particular lamp employed, a current of 200 milliamperes has proved to be a suitable one and has been everywhere used except where otherwise specified. If desired, the depth of the suspension may be kept constant and the current measured which under the given conditions makes the filament visible. This method, however, is inferior to the one adopted in being less sensitive and in involving more difficult calibrations.

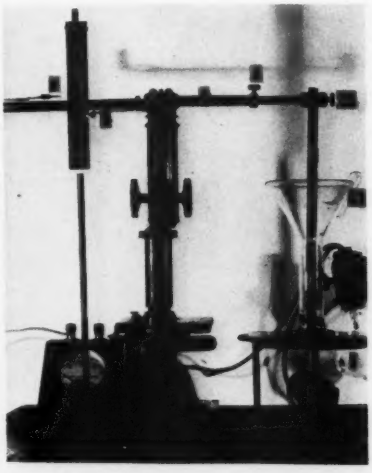

FIG. 1. A simple form of the apparatus, described in detail in the text.

A form of the apparatus somewhat simpler than the one actually employed, but which shows more clearly in a photograph its most essential parts is illustrated in Fig. 1. The image of the filament, 1, is reflected upward by the mirror, 2, through the vessel, 3, in which the

suspension to be examined is placed. This vessel is in the form of a tube 2.5 cm. in diameter with a funnel-like expansion above and closed below by a glass plate cemented to the tube with deKhotinsky cement. In cases where it is not necessary to keep the whole apparatus in a water bath for temperature control a separate glass funnel may be substituted.

Into the vessel, 3, plunges a tube, 4, coated internally with a dead-black varnish and closed at its lower end by a small coverglass cemented to it with deKhotinsky cement. It is best always to cover with paraffin any such cement which can come in contact with the solutions used in the experiments. The position of the plunger, 4, is adjusted by means of the rack and pinion of an ordinary microscope, 5, to the tube of which it is attached by the arm, 6. Attached to the microscope are also the pointer, 7, which gives readings on a millimeter scale and the writing point, 8, which touches the smoked paper of a kymograph (not shown). Any movement of 4 is therefore recorded by the kymograph, while at the same time its exact setting can be read from the scale. At the beginning of an experiment the apparatus is adjusted so that a scale reading of zero corresponds to close contact between the bottom of 3 and that of 4. The necessary adjustments of 4 are facilitated by the screws, 9 and 10. Where greater simplicity is desired, a satisfactory substitute for the arm, 6, can be improvised from several ordinary metal clamps.

Since in osmotic experiments on the erythrocyte (as will be pointed out elsewhere) accurate temperature control is essential, the vessel, 3, is usually immersed almost to the top of the funnel in a covered water-bath (not shown in Fig. 1) with blackened interior to cut off all light except that passing through a glass window in its bottom. For the design of this water-bath and for several other features of the apparatus the author is indebted to his assistants, Mr. Arthur K. Parpart and Mr. Wilbur A. Smith.

When the apparatus has been set for a series of experiments it is desirable not to disturb it in changing solutions. This is easily avoided by emptying the vessel, 3, through a removable glass tube (not shown) attached to a filter pump. Another fine-pointed glass tube, also not shown, is usually allowed to dip into the solution in 3. This tube is connected with the compressed air supply and provides in short experiments for rapid and uniform mixing of the blood and the solutions introduced into 3, while in longer ones the current of air may be used as desired to prevent any settling of erythrocytes on the bottom of the vessel. Other tubes connected with the compressed air supply and also not shown provide for the stirring of the water in the water-bath and the prevention of condensation of moisture on the window in its bottom when it is employed at low temperatures.

In using the instrument in an ordinary hemolysis experiment, the procedure is as follows. The tube, 4, is elevated until it is considerably above the position of the expected initial reading. The desired quantity of blood (usually one carefully formed drop from a special pipette) is placed on a small removable paraffin-coated shelf, 11, which is suspended from the side of the funnel. The kymograph is started and the compressed air turned on. Then, as the solution is suddenly poured upon the blood with one hand, the tube is lowered by the other until the image of the filament just appears. The beginning of the experiment is therefore shown by a sudden drop in the line made by the writing point. When the image is seen to increase slightly in brightness, the tube is quickly raised a few millimeters, causing it to disappear. When it again appears the tube is again raised, and this process is repeated until the tube emerges from the liquid. With the apparatus employed by the author and with 25 c.c. of liquid, which is a convenient quantity, this occurs at a scale reading of approximately 60 mm. representing, for samples of blood, in the proportions used, between 80 and 90 per cent apparent hemolysis.

FIG. 2. Typical record of the course of hemolysis of ox blood in 2M ethylene glycol. The time intervals are 5 seconds with every twelfth signal omitted.

The type of record obtained in an experiment of this sort is illustrated in Fig. 2. This particular record gives 11 points on a curve representing hemolysis of ox blood in 2M ethylene glycol. The time intervals marked on the record are of five seconds each with every twelfth one omitted. The slow fall of the curve prior to the sudden rise which indicates hemolysis is due to the gradual recovery by the erythrocytes, with the penetration of the solute, of their initial volumes, and their subsequent further swelling, after a pronounced shrinking

has been produced by the concentrated solution employed. Even with solutions of penetrating substances isosmotic with blood, the swelling that precedes hemolysis is usually indicated by a slight fall in the curve. By using greater dilutions of blood so that the readings appear higher on the scale these effects can be considerably magnified and used to

FIG. 3. Typical record of the partial hemolysis of ox blood in 0.082M NaCl. The marks on the curve indicate 30 second intervals.

good advantage in studying volume changes rather than hemolysis. As would be expected, swollen corpuscles produce lower and shrunken ones higher readings than normal ones, a fact already noted by Holker (1921).

In experiments of longer duration, where the method described is wasteful of kymograph paper and fatiguing to the eye of the observer, it is preferable to make readings at regular intervals marked by the writing point itself, allowing the drum to move only enough each time to record the level of the reading. A record of this sort covering 18.5 minutes with readings every 30 seconds is reproduced in Fig. 3. It represents the partial hemolysis of ox corpuscles in 0.082M NaCl slightly buffered for pH 7.4 with phosphates.

When the duration of the experiments is very short, *i.e.*, less than perhaps 10 seconds, kymograph records become difficult to make. Fairly complete and accurate hemolysis curves may be obtained, however, in such cases by setting the instrument in advance at any selected point and determining with a stop-watch the time required to reach this point. The vessel is then emptied and the experiment repeated with a different setting of the instrument, and so on, as many times as desired. The complete curve may then be plotted from the separate points obtained.

With experiments of such extremely short duration (*i.e.* less than

perhaps 1.5 seconds) that the time required for the uniform mixing of the blood and the solution becomes significant, it is scarcely profitable to attempt to obtain times corresponding to the lower scale readings. Fair accuracy, however, may be secured with sufficiently high settings so that most of the suspension is under the bottom of the inner tube, in which case imperfect mixing is much less serious than otherwise. It is for this reason, as well as because of the fact that the accuracy of the instrument is greater for the higher scale readings, that the author has chosen 75 per cent apparent hemolysis of an approximately 1 : 500 suspension as a very convenient criterion for comparison when for any reason it is necessary to select some single degree of hemolysis for this purpose. With the apparatus used and with most samples of blood this point usually corresponds to a scale reading in the vicinity of 40 mm.

In the use of the instrument several precautions may be mentioned. The only subjective feature of the method is the decision by the observer as to when the filament may be said to be visible. This decision is made with different degrees of readiness and constancy by different persons. The author finds it most convenient so to place the lamp and the mirror that what is seen in the tube is a single small loop of the filament. A reading is taken when the exact form of the entire loop is visible. To secure the greatest sensitiveness of the eye, readings should always be approached from the side of the invisibility rather than from that of the visibility of the filament. In any case, it is important to work fairly rapidly. The image should be approached without hesitation and the reading made without an attempt by moving the tube up and down unnecessarily to secure exactly the right degree of distinctness. What might otherwise be gained in this way is more than lost by the changes that are caused in the sensitiveness of the eye of the observer.

In general, the experience of each individual will soon teach him under what conditions he can secure the most reproducible results. Fortunately, the method permits no possible bias to enter into the measurements, since the observer is unable while making a reading to see the record on the drum, which reproduces with strict fidelity the results of his judgment. It is therefore a very simple matter for anyone using the method to obtain in this way, on a drum moved for the purpose by hand, a series of readings at different levels, which, when subsequently measured, will give exact information as to the reliability of his readings. The readings of the author, in a test of this sort, rarely show a variation of more than 0.3 mm. for a scale reading of 10 mm. or of more than 1.5 mm. for a scale reading of 50 mm. For a sus-

pension whose initial reading is 10 mm. these variations correspond to differences in the estimated percentages of hemolysis of approximately three and less than one per cent, respectively. By averaging a number of readings for a single point, such errors can be still further reduced. The method is therefore seen to be capable of yielding results of a high degree of accuracy.

IV.

The question of the relation between the observed scale readings and the corresponding degrees of hemolysis may now be discussed. Changes in the opacity of the suspension are due primarily to changes in the number of cells which it contains and secondarily to changes in

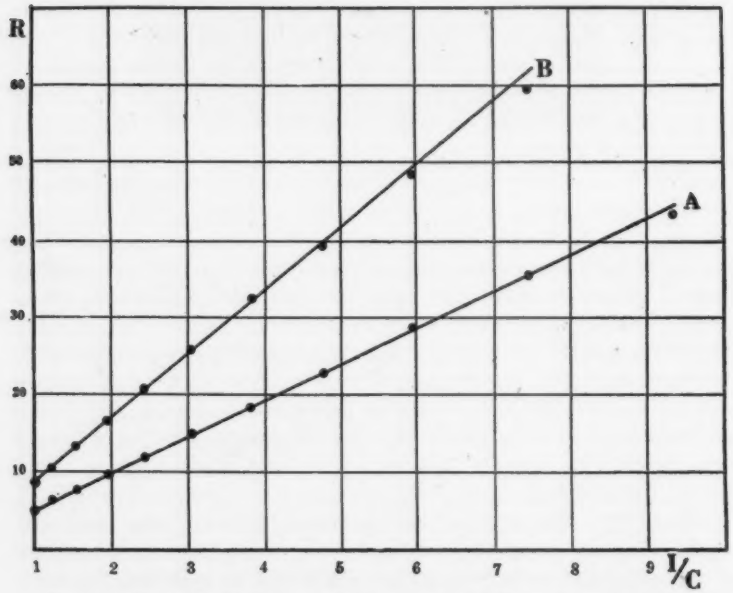

FIG. 4. Effect of dilution of blood on scale reading. Curve *A* represents dilution with 0.9 per cent NaCl of a suspension of ox erythrocytes. Curve *B* represents dilution of a similar but originally less concentrated suspension (approximately 1:500) with a solution containing hemoglobin in the proper amount to give standards representing different degrees of apparent hemolysis; R = scale reading in millimeters and $1/C$ = reciprocal of concentration in arbitrary units.

the properties of the individual cells and of the surrounding medium. Since the first mentioned factor is by far the most important, it may be considered first by itself as uncomplicated by, for example, the state

of swelling of the cells or the presence of hemoglobin in the surrounding solution.

The relation between the concentration of cells in a given suspension and the scale reading of the instrument may readily be obtained by a simple calibration experiment in which a geometrical series of dilutions of an original suspension is used for purposes of standardization. For example, beginning with 125 c.c. of a fairly concentrated suspension of cells in 0.9 per cent NaCl, 25 c.c. are removed for the first measurement and are replaced by 25 c.c. of the salt solution. After thorough mixing this process is then repeated for any desired number of times, a series of suspensions each four-fifths as concentrated as the one preceding it being obtained. Frequently, a factor of dilution of three-fourths or even one-half will give results which are entirely satisfactory with correspondingly less labor.

If now the scale readings so obtained are plotted against the reciprocals of the concentrations, as has been done in the graph labelled *A* in Fig. 4, it will be seen that the points lie almost on a straight line, indicating that the relation between the scale reading R, and the concentration C may be represented approximately by the rectangular hyperbola,

$$CR = \text{a constant.}$$

Actual calculations show that the errors introduced by estimating the relative number of cells in a given suspension, as compared with a standard, by means of this simple relation are usually insignificant. Thus, Table I, from which the data used in constructing graph *A* of Fig. 4 were obtained, shows in columns 1 and 4, respectively, the relative concentrations of cells as determined by actual dilution and as calculated from the relation,

$$CR = R_0,$$

R_0 being the scale reading for the original suspension whose concentration is taken as unity.

It will be observed that the differences amount in no case to more than one per cent, though in other similar experiments differences of two per cent, or rarely more, have been obtained. In general, the differences are greater for low scale readings where the errors of observation are relatively large. Because of the important effect of slight errors in determining the initial scale reading, R_0, an average value for the constant in the equation may, if desired, be obtained from all of the CR products. For comparison with the figures already mentioned there are given in column 5 concentration values calculated in this way. On the whole, they are seen to agree very closely with the values in columns 1 and 4.

As to the simple mathematical relationship found to exist between the number of cells and the observed scale-reading, it may be stated that much the same relation has been reported by Vlès (1921) and Holker (1921) who used methods somewhat similar in principle to the present one, though differing from it in a number of respects, for measuring the opacity of various cell suspensions.

TABLE I

Relation between Scale Readings and Concentration of Erythrocytes in Suspensions

Concentration in arbitrary units = C	Scale readings in millimeters = R (each figure is the average of 10 readings)	Product CR*	Concentration calculated from initial reading R_0 = 5.0	Concentration calculated from average of CR products = 4.85
1.00	5.0	5.0	—	—
.80	6.3	5.0	.79	.77
.64	7.7	4.9	.65	.63
.51	9.6	4.9	.52	.51
.41	12.0	4.9	.42	.40
.33	15.0	5.0	.33	.32
.26	18.3	4.8	.27	.27
.21	22.6	4.7	.22	.21
.17	28.6	4.8	.17	.17
.13	35.3	4.7	.14	.14
.11	43.6	4.7	.11	.11

* This product was calculated from more accurate values of C than those in column 1, which are rounded off to two places of decimals only.

The exactness with which relative numbers of cells can be estimated from scale readings, either by calculation or by the use of appropriate standards, particularly for readings above 20 mm., suggests the possibility of using the apparatus, though it was designed primarily for studies of hemolysis, for making the ordinary red-cell counts so frequently needed in physiological and in medical work and for which the laborious and not very accurate hemocytometer method is commonly employed. Preliminary experiments in this direction have shown that by first diluting the blood so that the resulting suspension gives a reading on the more sensitive part of the scale, successive independent determinations differing from one another by no more than one or two per cent may be obtained at will. The time required for each determination, exclusive of that required for cleaning and drying the blood pipette is approximately 15 seconds. With the enormously more laborious hemocytometer method, successive counts, as is well known, usually vary by at least five per cent. Of course, the method gives only relative and not absolute numbers of cells (though it can be made

absolute within the limits of the hemocytometer method itself by means of one preliminary cell count) and the readings obtained with it are affected by any variation in the size and shape of the erythrocytes in different samples of blood, as well as by their numbers. The errors to be expected from these sources, however, under the usual physiological conditions are not likely to be as great as those constantly and unavoidably associated with the far more difficult method now almost universally employed.

In using the instrument to estimate percentage of hemolysis, several factors in addition to the concentration of cells must be considered. In the first place, during hemolysis not only do the cells decrease in number, but the hemoglobin liberated from them and contained in the surrounding solution absorbs light and therefore tends to produce lower scale readings than correspond to the mere number of cells. This complication may be dealt with readily, however, by making the dilutions in the calibration series with a solution containing the concentration of hemoglobin that would result from complete hemolysis of the cells. Such a solution is readily prepared by adding to distilled water twice the quantity of blood contained in the same volume of the standard suspension and then, after complete hemolysis has occurred and the solution is entirely transparent, mixing with it an equal volume of sodium chloride solution of twice the concentration of that desired.

As a matter of fact, it turns out that with the dilution of blood that is otherwise most convenient to work with (approximately one part of blood to five hundred of solution) the effect of the hemoglobin on the reading of the instrument, while detectable, is, practically, almost negligible. Under these circumstances the product:

$$(100 - \text{per cent hemolysis}) \times \text{scale reading}$$

proves to be almost constant, as is indicated in graph B of Fig. 4 where the scale readings plotted against the reciprocals of the percentage of unhemolyzed cells lie almost on a straight line.

The theoretical apparent percentages of hemolysis represented in the prepared standards in this particular experiment and the corresponding figures as calculated by the equation

$$\text{per cent hemolysis} = 100\left(1 - \frac{R_0}{R}\right)$$

are given in Table II in columns 1, 3 and 4, respectively, and are seen to be in better agreement than might, from the nature of the case, reasonably have been expected.

For many purposes, therefore, with a very fair degree of accuracy,

apparent percentages of hemolysis may simply be calculated from initial scale readings as if the presence of hemoglobin in the external solution could be disregarded. For such calculations, a graphic method, which perhaps requires no explanation here, has been found to save much time. In cases where higher concentrations of erythrocytes are employed or where special accuracy is required, however, appropriate standards for calibration should be prepared.

TABLE II

Relation between Scale Readings and Apparent Percentages of Hemolysis

Apparent percentage of hemolysis represented by standard	Scale reading (each figure is the average of 5 readings)	Percentage hemolysis calculated from initial scale reading	Percentage hemolysis calculated from average of *CR* products
0	8.6	—	—
20	10.4	17	20
36	13.0	34	36
49	16.6	48	50
59	20.6	58	60
67	25.7	67	68
74	32.2	73	74
79	39.2	78	79
83	48.5	82	83
87	59.6	86	86

The assumption has so far tacitly been made that a given per cent of hemolysis may be represented by a mixture of unaltered cells and of completely hemolyzed cells. This is, unfortunately, not strictly true. In the first place, any solution which is sufficiently dilute to cause osmotic hemolysis of any of the cells must of necessity cause swelling of all of the unhemolyzed cells. In the second place, the possibility must be considered that cells which have not as yet undergone hemolysis may have, nevertheless, given up some of their hemoglobin to the surrounding solution. Both of these factors might be expected to have optical effects which would considerably complicate the situation as so far outlined.

With regard to the first factor, an approximate allowance may readily be made for it by taking as the initial reading for purposes of calculation, not that for a given suspension in 0.9 per cent NaCl, but that for a similar suspension in a solution which is decidedly hypotonic though not quite sufficiently so to cause any hemolysis. This concentration may readily be determined by experiment; for ox blood it is usually in the vicinity of $M/8$ NaCl. Figures obtained in this way by calculation or by calibration with standards made up as before but with the use of hypotonic instead of isotonic solutions, are undoubtedly

more accurate than those secured with the neglect of this precaution. It is impossible, however, because of the heterogeneous nature of the material dealt with, to prepare by a simple method of mixtures standards which reproduce with complete fidelity the conditions in a partly hemolyzed sample of the blood.

Even more troublesome is the second difficulty mentioned above. If osmotic hemolysis is, as is maintained by Saslow (1929) an "all or none" phenomenon, then the preparation of standards representing fairly well a given degree of hemolysis is perhaps possible. If, on the other hand, as is believed by Báron (1928), this is not the case, but in a given mixture of hemolyzing cells some have undergone complete hemolysis (in the sense of becoming completely invisible), while others have lost lesser amounts of hemoglobin which can be expected to vary greatly with the conditions of the experiment, then not only is it impossible to prepare standards representing accurately different percentages of hemolysis, but the term percentage of hemolysis itself ceases to have any very exact meaning.

Under these circumstances, and until there is more general agreement than there is at present as to whether osmotic hemolysis is or is not an "all or none" phenomenon, it is perhaps unprofitable to try to introduce into our methods refinements which may have little real significance. It seems preferable merely to speak, as has already been done, of an apparent or an approximate percentage of hemolysis, using for our estimations some convenient though arbitrary type of standard. Figures of this sort will have a considerable value, if used with a recognition of their limitations. In any case, regardless of the type of standard employed, such figures will usually involve an uncertainty of only a few per cent in the assumed degree of hemolysis.

In the absence of any general agreement at present as to a precise definition of percentage of hemolysis, the especial value of a method such as the one here described becomes apparent. The kymograph tracings obtained with it are exact and unequivocal. There may be doubt as to whether a certain point on the record indicates 75 per cent or 78 per cent hemolysis, but the point itself is not in doubt. In most experiments what is desired is not so much to know how long it requires to reach, for example, exactly 75 per cent hemolysis, assuming that this expression has any precise meaning, but rather how long it requires under the chosen conditions to reach a point on the hemolysis curve which can be represented by some reproducible standard. This is possible with the present method with a high degree of accuracy.

SUMMARY

1. A simple method is described by which it is possible to measure with a very satisfactory degree of accuracy the rate of hemolysis where the time involved exceeds approximately one second. If the duration of the experiment is ten seconds or more, a complete graphic record of the entire process up to an apparent degree of hemolysis of between 80 and 90 per cent may be obtained.

2. The method may also be used for the accurate determination of the relative numbers of erythrocytes in different suspensions and, assuming a satisfactory definition for the expression "percentage of hemolysis," for the rapid estimation of the latter, within the range most useful for experimental purposes, with an error of no more than one or two per cent.

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